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TITLE: Thio and Seleno Rhodamine Derivatives as Reversal Agents of Multidrug Resistance in Breast Cancer

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14. ABSTRACT

The overall objective of this proposal is to identify rhodamine analogues that photosensitize the inhibition of Pgp function in the presence or absence of modulators and/or chemotherapeutic agents. The specific aims are: 1) the synthesis of a series of rhodamine analogues and the determination of their photophysical properties using standard methods, 2) the measurement of uptake and/or efflux of these analogues in the presence or absence of Pgp modulators in chemo-sensitive and Pgp-expressing cell lines, 3) the determination of doxorubicin or Calcein AM uptake in chemo-sensitive and resistant cell lines in the presence or absence of rhodamine analogues with or without light exposure, and 4) the determination of whether rhodamine-induced photosensitized inhibition of Pgp results in greater chemo-sensitivity and/or enhanced phototoxicity. The completed work demonstrates that substituent effects among the various rhodamine analogues impact their phototoxicity towards either chemosensitive AUXB1 cells or multidrug resistant CR1R12 cells. The substituent effects for the rhodamine analogues were realized in drug resistant CR1R12 cells only when the Pgp modulator verapamil was present indicating that the substituent effects extend to the interaction between the chemical structure of the analogues and binding/transport function of Pgp. The presence of vanadate, oligomycin, or verapamil increased the level of uptake of three dyes into CR1R12 cells. Presumably, the additives impair the ability of Pgp to remove the rhodamine analogues from the cells. Following treatment with verapamil, the mitochondria are targets for the photosensitizers in the multidrug-resistant CR1R12 cells.

15. SUBJECT TERMS
Photodynamic therapy, photosensitizers, multidrug resistance, P-glycoprotein

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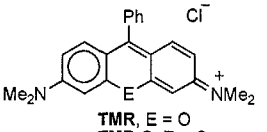
Thio and Seleno Rhodamine Derivatives as Reversal Agents of Multidrug Resistance in Breast Cancer

Introduction

Multidrug resistance (MDR), has been identified as a major impediment to successful chemotherapy for a variety of cancers.[1] Multidrug resistance is attributed to the action of a class of membrane proteins termed the ATP binding cassette (ABC) transporters.[1-3] These proteins, P-glycoprotein (Pgp), the multidrug resistance protein (MRP) series, breast cancer resistance protein (BCRP) and lung resistance-related protein (LRP) are involved in the transport of substrates that span a wide range of chemically diverse and structurally disparate compounds including a majority of the commonly prescribed chemotherapeutic agents.[4] The ABC transport proteins possess similarities such as their dependence on the energy derived from ATP hydrolysis to transport substrates,[5] the presence of units of 6 transmembrane domains (TMDs) which are poorly conserved among the different proteins, and the presence of nucleotide binding domains (NBDs) which are highly conserved.[1-3] The majority of the ABC transporters are responsible for the transmembrane movement of ions and compounds in order to maintain levels necessary for cell viability. The drug resistance proteins in this class, of which P-glycoprotein is reportedly the most prevalent,[6] have the unique ability to pump a broad array of chemotherapeutics actively out of the cells, dramatically reducing drug concentration and thus their therapeutic effectiveness.[1,6] Interestingly, expression of Pgp and its recognition of various substrates can be induced by exposure to a single compound or therapeutic agent.[7] Due to the diverse nature of the molecules transported by Pgp, its function as an efflux pump is of major concern for clinicians and under intense investigation by numerous groups worldwide.[8]

Attempts have been made to alleviate the function of Pgp using modulators such as verapamil, quinidine, cyclosporin A, PSC833 and others.[1,9] These modulators bind to the drug transport binding site(s) on the protein and impede the binding and subsequent efflux of agent(s) of interest thus allowing their accumulation.

A number of MDR reversal agents including verapamil, cyclosporin A, and PSC833, have been examined to counteract the mechanisms of drug resistance.[3] Major drawbacks for these compounds are their effects on cell metabolism and their toxicity toward normal tissues. The cationic rhodamine dyes, such as rhodamine 123 (Rh123) and tetramethyl rosamine (TMR), are transport substrates for Pgp but do not inhibit Pgp.[4,5] Pgp-mediated efflux of these highly fluorescent molecules has been used to determine the efficacy of Pgp modulators. We were examining thio- and seleno-analogues of TMR as transport substrates for Pgp and found, unexpectedly, that Pgp function was inhibited in dye-treated cells upon exposure to light as measured by uptake of adriamycin or Calcein AM. This inhibition of Pgp was not observed in the absence of light and was not observed in irradiated, TMR-treated cells. This unexpected result suggests that photosensitized inhibition of Pgp and other efflux pumps might be used to reverse MDR in breast cancer.



TMR, E = O
TMR-S, E = S
TMR-Se, E = Se

Chart 1. Structure of tetramethylrosamine (**TMR**) and thio- (**TMR-S**) and seleno-analogues (**TMR-Se**).

Studies of the efflux function of Pgp have traditionally used Pgp transport molecules that are either intrinsically fluorescent or are tagged with a fluorescent marker.[10,11] Assays employing such compounds usually monitor the efflux of a specific molecule, such as Rhodamine 123, from cells by measuring a decrease

in intracellular fluorescence.[11] In addition, the quenching of tetramethylrosamine (**TMR**) fluorescence has been used to measure transport by Pgp in isolated membrane vesicles.[12]

We have been studying heavy-chalcogen analogues of the rhodamine dyes and **TMR** (Chart 1) for their utility as phototoxic agents for malignant cells and tissues.[13,14] These compounds have an affinity for mitochondrial localization in cancer cells due to their delocalized positive charge. The insertion of the heavy chalcogen atoms sulfur, selenium or tellurium into these compounds, replacing oxygen in the rhodamines and **TMR**, allows for higher triplet yields and, consequently, higher quantum yields for the generation of singlet oxygen ($^1O_2$). The generation of $^1O_2$ results in damage to intracellular structures and functions ultimately leading to cell death. The use of photosensitizers in combination with Pgp modulators can reduce toxicity toward normal tissues during treatment of multidrug-resistant cancers since the activating light for the photosensitizer can be delivered specifically to the areas of treatment. We extend our initial studies of thio-tetramethylrosamine (**TMR-S**) and seleno-tetramethylrosamine (**TMR-Se**) as photosensitizers for both chemosensitive and multidrug-resistant cells, as transport molecules for Pgp, and as inhibitors of Pgp-mediated multidrug resistance[15] to include a series of structurally related derivatives. In this report, we compare the uptake and phototoxicity of a series of thio-tetramethylrosamine and seleno-tetramethylrosamine analogues in chemosensitive AUXB1 cells and in multidrug-resistant, highly Pgp-expressing CR1R12 cells in which Pgp function has been inhibited by the addition of verapamil. In the presence of verapamil, several of the thio-tetramethylrosamine derivatives are effective photosensitizers for multidrug-resistant CR1R12 cells.

The overall objective of this proposal was to identify thio- and seleno-rhodamine analogues that photosensitize the inhibition of Pgp function in the presence or absence of modulators and/or chemotherapeutic agents. The specific aims are: 1) the synthesis of a series of thio- and seleno-analogues of TMR, Rh123, and other rhodamines and the determination of their photophysical properties using standard methods, 2) the measurement of uptake and/or efflux of these analogues in the presence or absence of Pgp modulators in chemo-sensitive and Pgp-expressing cell lines, 3) the determination of doxorubicin or Calcein AM uptake in chemo-sensitive and resistant cell lines in the presence or absence of rhodamine analogues with or without light exposure, and 4) the determination of whether rhodamine-induced photosensitized inhibition of Pgp results in greater chemo-sensitivity and/or enhanced phototoxicity.

Body

Original Statement of Work and Summary of Accomplishements

Task 1. Synthesize thio and seleno rhodamine derivatives and test photochemistry.

a. Modify side chain substituents by adding phenyl, carboxylic acid, methyl groups etc.

b. Determine hydrophobicity via octanol/water partioning, fluorescence, triplet and singlet oxygen yields using standard methods.

- 10 new derivatives were prepared and characterized for values of log P, fluorescence, and singlet oxygen yields as described in: Gibson, S. L.; Holt, J. J.; Ye, M.; Donnelly, D. J.; Ohulchanskyy, T. Y.; You, Y.; Detty, M. R. "Structure-activity Studies of Uptake and Phototoxicity with Heavy-chalcogen Analogues of Tetramethylrosamine *in vitro* in Chemosensitive and Multidrug-resistant Cells," *Biorg. Med. Chem.* **2005**, *13*, 6394-6403, a copy of which may be found in the Appendix.

Task 2. Determine the uptake and/or efflux of rhodamine analogues in cultured chemo-sensitive and resistant cells. Months 1-3.

a. Initiate cultures of the Chinese hamster ovary chemo-sensitive cell line AUXB1 and its highly Pgp expressing subline CR1R12 and the chemo-sensitive human breast cancer cell line MCF-7 and its resistant subline MCF-7/ADR (doxorubicin resistant).
b. Study the uptake of rhodamine analogues (0.1-10uM) by fluorescence in chemo-sensitive or resistant cell lines in the presence or absence of Pgp modulators verapamil (0-70uM), cyclosporin A (0-7uM) or PSC833 (0-50uM).

- Three derivatives had sufficient fluorescence to allow direct measurement of intracellular uptake. The uptake of **TMR-S**, and the *p*-MeO and *m*-MeO analogues were evaluated in AUXB1 cells and in CR1R12 cells in the presence or absence of the Pgp modulator verapamil as described in: Gibson, S. L.; Holt, J. J.; Ye, M.; Donnelly, D. J.; Ohulchanskyy, T. Y.; You, Y.; Detty, M. R. "Structure-activity Studies of Uptake and Phototoxicity with Heavy-chalcogen Analogues of Tetramethylrosamine *in vitro* in Chemosensitive and Multidrug-resistant Cells," *Biorg. Med. Chem.* **2005**, *13*, 5927-5935, a copy of which may be found in the Appendix.

Task 3. Determine uptake of the Pgp transport substrates doxorubicin or Calcein AM, using fluorescence, in chemo-sensitive and resistant cell lines. Months 4-8.
a. Uptake and/or efflux of doxorubicin or Calcein AM, transport substrates for Pgp, will be monitored in chemo-sensitive or resistant cell lines in the dark in the presence or absence of rhodamine analogues.
b. Uptake and/or efflux of doxorubicin or Calcein AM in cell lines after exposure to rhodamine analogues and light.

- Three rhodamine derivatives were monitored for uptake in the presence of Calcein AM, oligomycin, and vanadate.

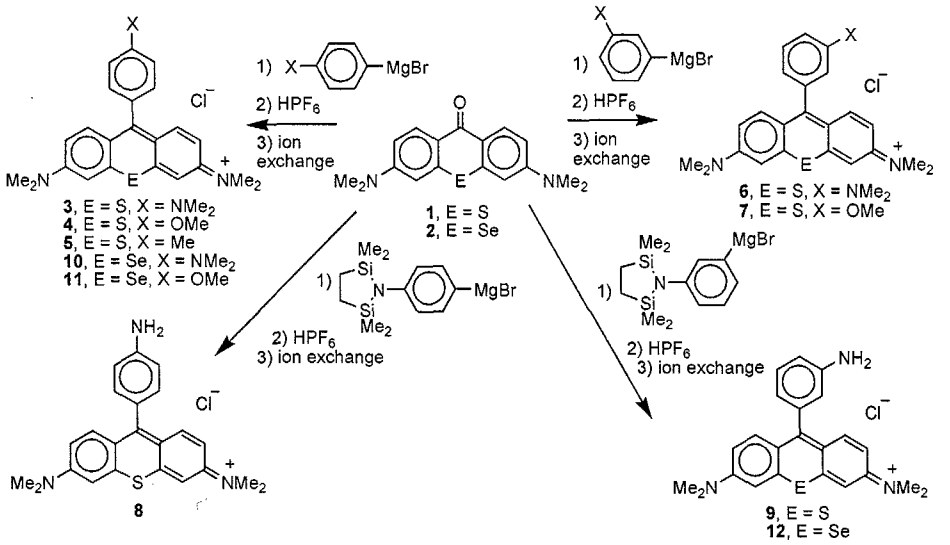
Task 4. Measure photo-induced cytotoxicity and photo-induced effects on chemotoxicity of rhodamine analogues in chemo-sensitive and resistant cell lines. Months 8-12.
a. Determine the photoxicity of rhodamine analogues towards chemo-sensitive and resistant cell lines.
b. Measure chemotoxicity of doxorubicin after exposure of chemo-sensitive and resistant cell lines to rhodamine analogues in the dark or after light exposure. Months 10-12.

- The photo-induced cytotoxicities and the inherent dark toxicities of 12 different rhodamine analogues toward chemosensitive AUXB1 cells and drug-resistant CR1R12 cells were measured in the presence and absence of verapamil. Details are provided as described in: Gibson, S. L.; Holt, J. J.; Ye, M.; Donnelly, D. J.; Ohulchanskyy, T. Y.; You, Y.; Detty, M. R. "Structure-activity Studies of Uptake and Phototoxicity with Heavy-chalcogen Analogues of Tetramethylrosamine *in vitro* in Chemosensitive and Multidrug-resistant Cells," *Biorg. Med. Chem.* **2005**, *13*, 5927-5935, a copy of which may be found in the Appendix.

Results

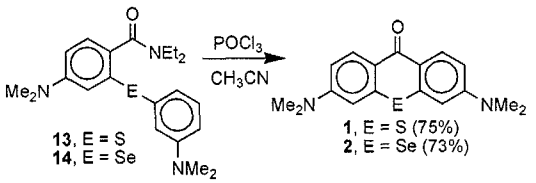
Synthesis of Heavy-chalcogen Analogues of the Rhodamines. The synthetic approach to the synthesis of **TMR-S** and **TMR-Se** involves the addition of phenylmagnesium bromide to chalcogenoxanthen-9-ones **1** and **2**, respectively, followed by acid-induced dehydration and ion exchange.[14] In order to investigate structure-activity relationships within a series of related molecules, the chalcogenoxanthylium dyes **3-12** of Scheme 1 were prepared via a similar approach. The Grignard reagents prepared from 3-bromo or 4-bromo-derivatives of various substituted benzenes were added to the chalcogenoxanthen-9-ones **1** and **2**.[14,16] Dehydration with

AcOH/HPF_6 gave the PF_6^- salts of dyes **3-12**. Ion exchange with Amberlite IRA-400 chloride exchange resin gave the chloride salts **3-12** in 40-98% isolated yields overall.



Scheme 1. Synthesis of thio- and seleno-analogues of **TMR**.

The chalcogenoxanthen-9-ones **1** and **2** have been prepared in low yield from benzamide derivatives **13** and **14**, respectively, and strong base.[14,16] We have found that the yields are greatly improved by cyclization with $POCl_3$ in refluxing acetonitrile as shown in Scheme 2. Chalcogenoxanthones **1** and **2** were isolated in 75% and 73% isolated yields, respectively, with this approach.



Scheme 2. Synthesis of Chalcogenoxanthen-9-one **1** and **2**.

Values of absorption maxima, λ_{max}, and associated molar extinction coefficients, ε, as well as values of the *n*-octanol/water partition coefficient(log *P*) are compiled in Table 1. Values of λ_{max} are not very sensitive to the nature of the substituent attached to the 9-phenyl substituent on the chalcogenoxanthylium nucleus (Table 1). The range of values of λ_{max} for the thio **TMR** analogues is 565-573 nm while the range of values for the seleno **TMR** analogues is 576-582 nm.

Values of the *n*-octanol/water partition coefficient, log *P*, are more sensitive to substituent effects with a range from -0.37 for 3-aminophenyl seleno derivative **12** to 1.13 for 3-*N,N*-dimethylaminophenyl thio derivative **6**. The *N,N*-dimethylamino-substituted derivatives **3**, **6**, and **10** and 4-methylphenyl derivative **5** were the most lipophilic dyes in the series. Methoxy-substituted derivatives **4**, **7**, and **11**, **TMR-S**, **TMR-Se**, and amino-substituted derivatives **8**, **9**, and **12** were far more hydrophilic with values of log *P* near 0.

Quantum yields for the generation of singlet oxygen [$\phi(^1O_2)$] by direct detection of singlet oxygen[17] and quantum yields for fluorescence (ϕ_F) were measured for **3-12** in $CHCl_3$ and MeOH and values are compiled in

Table 2. Substituents on the 9-phenyl ring had a pronounced effect on values of $\phi(^1O_2)$ and ϕ_F. Values of both $\phi(^1O_2)$ and ϕ_F are low (≤ 0.04) for derivatives containing the *N,N*-dimethylamino substituent presumably due to increased rates of internal conversion in these systems. Even the seleno analogue **10** had a small value of $\phi(^1O_2)$ indicating that intersystem crossing was not competitive with internal conversion in these derivatives. Rates of internal conversion were not as dominant in the amino-substituted derivatives. Values of $\phi(^1O_2)$ were between 0.02 and 0.06 for thio derivatives **8** and **9** while values of ϕ_F were between 0.01 and 0.09. The 3-aminophenyl seleno derivative **12** gave $\phi(^1O_2)$ of 0.31 in $CHCl_3$.

Table 1. Absorption maxima (λ_{max}) and molar extinction coefficients (ε) in MeOH and *n*-octanol/water partition coefficients (log *P*) for **TMR-S**, **TMR-Se**, and **3-12.**

Compd	E	9-Ph-X	λ_{max} (nm), MeOH	ε (M^{-1} cm^{-1}), MeOH	log P^a
TMR-S	S	H	571	50,100	0.07 ± 0.02
TMR-Se	Se	H	582	69,200	–0.09 ± 0.09
3	S	4-NMe_2	573	72,400	0.94 ± 0.01
4	S	4-OMe	569	97,700	–0.09 ± 0.03
5	S	4-Me	570	95,400	0.81 ± 0.04
6	S	3-NMe_2	573	72,400	1.13 ± 0.02
7	S	3-OMe	571	87,100	0.14 ± 0.01
8	S	4-NH_2	565	75,800	0.20 ± 0.02
9	S	3-NH_2	571	51,000	0.05 ± 0.03
10	Se	4-NMe_2	576	56,200	0.49 ± 0.02
11	Se	4-OMe	576	56,200	0.18 ± 0.09
12	Se	3-NH_2	582	56,300	–0.37 ± 0.092

[a] pH 6.0 phosphate buffer as the aqueous phase.

With **TMR-S**, **TMR-Se**, methoxyphenyl derivatives **4**, **7**, and **11**, and 4-methylphenyl derivative **5**, internal conversion was small relative to the combined yields for $\phi(^1O_2)$ and ϕ_F (Table 2). For the thio derivatives **TMR-S**, **4**, **5**, and **7**, $\phi(^1O_2)$ ranged between 0.09 and 0.30 and ϕ_F ranged between 0.23 and 0.44. For seleno derivatives **TMR-Se** and **11**, $\phi(^1O_2)$ ranged between 0.53 and 0.87 while ϕ_F was ≤ 0.01. In general, values of $\phi(^1O_2)$ were higher in $CHCl_3$ relative to MeOH while values of ϕ_F were slightly higher in MeOH relative to $CHCl_3$.

Table 2. Quantum yields for the generation of singlet oxygen [$\phi(^1O_2)$] and for fluorescence (ϕ_F) in MeOH and $CHCl_3$ for **TMR-S**, **TMR-Se**, and **3-12**.

Compd	9-Ph-X	$\phi(^1O_2)$, MeOH[a]	$\phi(^1O_2)$, $CHCl_3$[a]	ϕ_F, MeOH[a]	ϕ_F, $CHCl_3$[a]
TMR-S	H	0.21 ± 0.01[b]	--	0.44 ± 0.01[b]	--
TMR-Se	H	0.87 ± 0.02[b]	--	0.005 ± 0.01[b]	--
3	4-NMe_2	0.01 ± 0.01	0.01 ± 0.01	0.003 ± 0.001	0.003 ± 0.001
4	4-OMe	0.09 ± 0.01	0.22 ± 0.01	0.27 ± 0.01	0.23 ± 0.01
5	4-Me	0.11 ± 0.01	0.26 ± 0.01	0.29 ± 0.01	0.23 ± 0.01
6	3-NMe_2	0.02 ± 0.01	0.04 ± 0.01	0.001 ± 0.001	0.001 ± 0.001
7	3-OMe	0.16 ± 0.02	0.30 ± 0.02	0.35 ± 0.02	0.28 ± 0.02
8	4-NH_2	0.02 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.07 ± 0.01
9	3-NH_2	0.04 ± 0.01	0.06 ± 0.01	0.01 ± 0.01	0.09 ± 0.02
10	4-NMe_2	0.02 ± 0.01	0.02 ± 0.01	0.001 ± 0.001	0.001 ± 0.001
11	4-OMe	0.60 ± 0.02	0.53 ± 0.02	0.01 ± 0.01	0.01 ± 0.01
12	3-NH_2	0.05 ± 0.01	0.31 ± 0.01	0.004 ± 0.001	0.004 ± 0.001

[a] ± standard deviation.

[b] Reference 14.

Intracellular Accumulation of Heavy-chalcogen Analogues of the Rhodamines. As shown in Table 2, thio-analogues **TMR-S**, **4**, and **7** were sufficiently fluorescent to allow quantitative determination of cellular uptake into AUXB1 and CR1R12 cells. Drug sensitive AUXB1 cells or multidrug resistant CR1R12 cells were exposed to various concentrations of **TMR-S**, **4**, or **7** in the dark for 2 h in complete medium. The data displayed in Figure 1 demonstrate that nearly identical uptake is observed for **TMR-S**, **4**, and **7** in the drug-sensitive AUXB1 cells and that 5-8 femtomole/cell of these dyes is retained by the cells when exposed to 1×10^{-5} M dye for 2 h. Addition of 5×10^{-5} M verapamil prior to dye exposure did not affect the uptake of these dyes into the AUXB1 cells (data not shown).

In contrast, exposure of the multidrug-resistant CR1R12 cells to 1×10^{-5} M **TMR-S**, **4**, or **7** for 2 h gave only 3-4 femtomole/cell as shown in Figure 2. However, when the CR1R12 cells have a 15 min prior exposure to 5×10^{-5} M verapamil, a 2- to 4-fold increase in intracellular dye accumulation was obtained as shown in Figure 2 for dye concentrations of 1×10^{-6} to 1×10^{-5} M.

The data in Figure 2 demonstrate that uptake of **TMR-S** or dye **4** in CR1R12 cells is significantly greater for all the concentrations tested than the uptake of compound **7**. *P* values range from <0.05 to <0.001 for the comparisons. It is interesting to note that uptake of **TMR-S**, **4**, and **7** into CR1R12 cells after exposure to 5×10^{-5} M verapamil is equivalent to or marginally greater than the uptake of these dyes into the drug sensitive AUXB1 cells (Figure 1 *vs.* Figure 2).

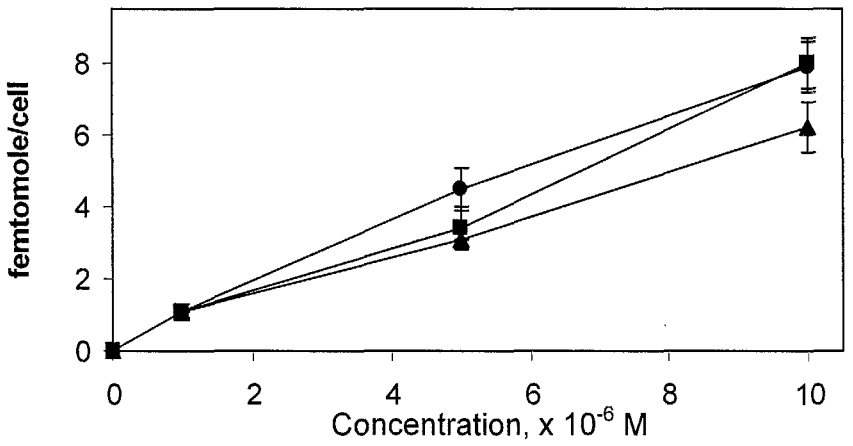


Figure 1. Intracellular accumulation of **TMR-S** (■) and thio derivatives **4** (●) or **7** (▲) with methoxy substituents into drug sensitive AUXB1 cells. Cell culture and dye exposure conditions and the method to determine the intracellular dye content are described in detail in the Experimental Section. Each data point represents the mean obtained from at least 3 separate experiments performed in duplicate, error bars are the SEM.

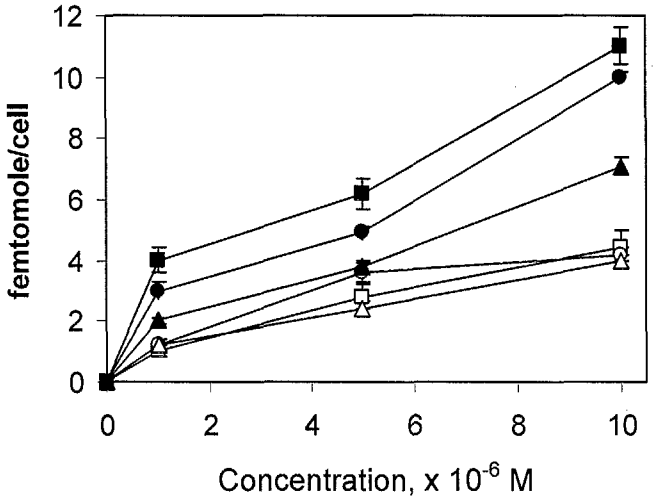


Figure 2. Intracellular accumulation of **TMRS** (□), **4** (○) or **7** (△) into cultured multidrug resistant CR1R12 cells and intracellular accumulation of **TMRS** (■), **4** (●) or **7** (▲) in cells exposed to 5×10^{-5} M verapamil prior to addition of dyes to cell monolayers. Cell culture and dye exposure conditions and the method to determine the intracellular dye content are described in detail in the Experimental section. Each data point represents the mean obtained from at least 3 separate experiments performed in duplicate, error bars are the SEM.

Phototoxicity of Thio-analogues of the Rhodamines towards AUXB1 and CR1R12 cells. In order to compare the phototoxicity of the heavy-chalcogen analogues of **TMR**, cultured chemosensitive AUXB1 and multidrug resistant CR1R12 cells were exposed to the compounds for 2 h in the dark and then irradiated with 5 J cm^{-2} broad band, 350-750-nm visible light. Dark controls were treated with dye for 2 h and not irradiated. The CR1R12 cells were treated with 5×10^{-5} M verapamil for 15 min prior to treatment with the thio-analogues of **TMR**. The data shown in Figure 3 depict the results obtained from chemosensitive AUXB1 cells that were exposed to **TMR-S** and the thio-tetramethylrosamine analogues **3-9**. In the absence of light, **TMR-S** and **3-9** gave >90% cell survival in AUXB1 cells at concentrations up to 1×10^{-5} M with or without added verapamil

(data not shown). The data obtained demonstrate that **TMR-S** and 4-tolyl derivative **5** were the two most phototoxic compounds with nearly identical phototoxicity toward AUXB1 cells and were phototoxic at all the concentrations tested. 4-Methoxyphenyl derivative **4** was the next most effective photosensitizer and was significantly more phototoxic than 3-methoxyphenyl derivative **7** at lower dye concentrations. However, all four of these compounds have equivalent phototoxicity at or above 1×10^{-6} M. Derivative **3** is only phototoxic at concentrations of 1×10^{-6} M or higher. Analogues **6**, **8**, and **9** demonstrated little or no phototoxicity toward AUXB1 cells at any concentration. These data indicate that, at least at lower concentrations, substituent effects impact phototoxicity in the thio-tetramethylrosamine derivatives.

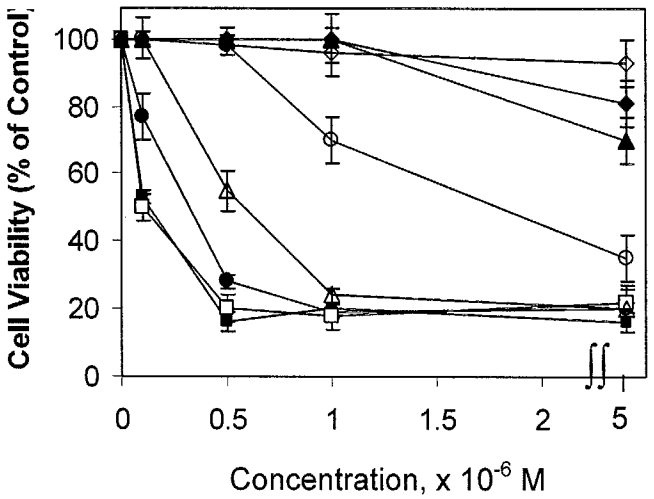


Figure 3. Phototoxicity of **TMR-S** and thio-tetramethylrosamine analogues **3-9** towards chemosensitive AUXB1 cells. The data depict the results obtained after chemosensitive AUXB1 cells were exposed to **TMR-S** (■), **3** (○), **4** (●), **5** (□), **6** (▲), **7** (△), **8** (◇), or **9** (◆). Cell culture and dye and light exposure conditions are described in detail in the Experimental Section. Each data point represents the mean of at least three separate experiments performed in duplicate, error bars are the SEM. Data are expressed as percent cell viability compared to control cells, those exposed to neither dye nor light.

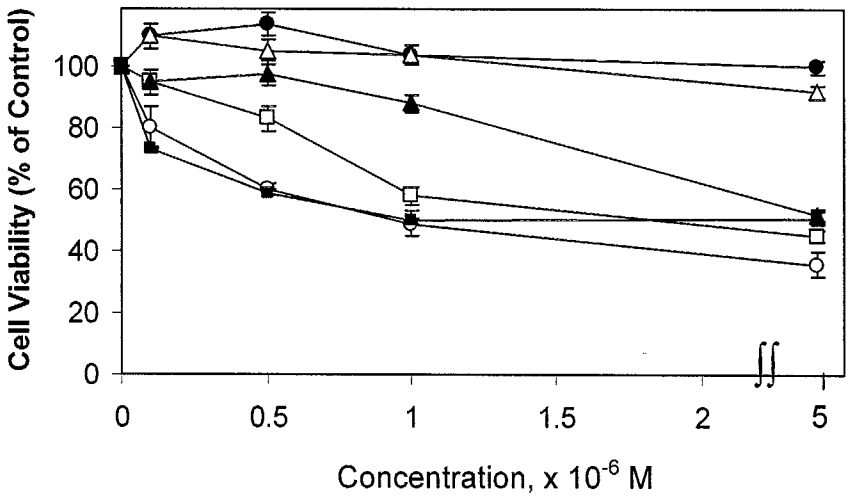


Figure 4. Phototoxicity of **TMR-S** (○), **3** (●), **4** (□), **5** (.■.), **6** (Δ), or **7** (▲) towards multidrug resistant CR1R12 cells following a 15-min incubation with 5×10^{-5} M verapamil. Cell culture and dye and light exposure conditions are described in detail in

the Experimental Section. Each data point represents the mean of at least three separate experiments performed in duplicate, error bars are the SEM. Data are expressed as percent cell viability compared to control cells, those exposed to neither dye nor light.

In the absence of prior exposure to verapamil, none of the thio analogues of **TMR** showed any significant dark or phototoxicity (>90% cell survival) toward CR1R12 cells at concentrations ≤ 5 x 10^{-6} M (data not shown). If the CR1R12 cells were treated with 5 × 10^{-5} M verapamil for 15 min prior to treatment with the photosensitizers, then substituent effects impacted photosensitizer potency. In the CR1R12 cells, none of the aminophenyl- or *N,N*-dimethylaminophenyl-substituted compounds – **3**, **6**, **8**, and **9** – showed any phototoxicity. However, the phototoxicity of **TMR-S** and the methoxy-substituted derivatives **4** and **7** towards these cells, (Figure 4) was consistent with their rank order of uptake: **TMR-S** > **4** > **7**, as shown in Figure 2. Derivative **5** was identical to **TMR-S** with respect to phototoxicity toward CR1R12 cells. In the presence of 5 × 10^{-5} M verapamil and in the absence of light, **TMR-S** and **3-9** gave >90% cell survival in CR1R12 cells at concentrations up to 1 × 10^{-5} M (data not shown). Comparison of cell viability following treatment of either AUXB1 or CR1R12 cells with **TMR-S**, **4**, **5**, or **7** and 5 J cm^{-2} of 350-750-nm light suggests that AUXB1 cells are more susceptible to photodamage than CR1R12 cells.

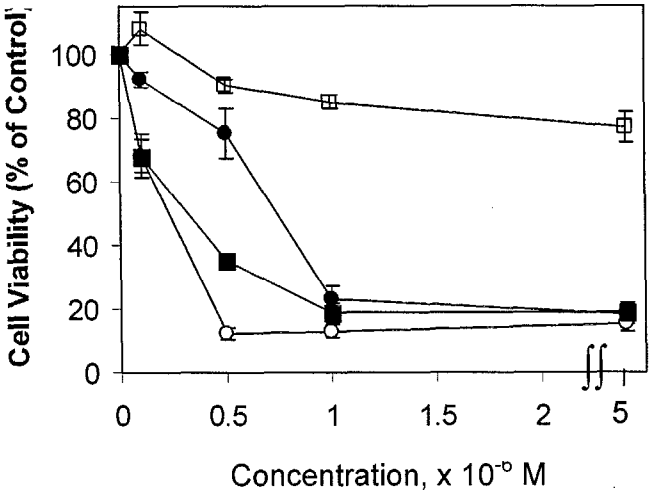


Figure 5. A comparison of the phototoxicity of **TMR-Se** (○) and seleno-tetramethylrosamine analogues **10** (●), **11** (■), and **12** (□). Cell culture and dye and light exposure conditions are described in detail in the Experimental Section. Each data point represents the mean of at least three separate experiments performed in duplicate, error bars are the SEM. Data are expressed as percent cell viability compared to control cells, those exposed to neither dye nor light.

Phototoxicity of Selenium-containing Rhodamine Analogues towards Chemosensitive AUXB1 Cells. The seleno tetramethylrosamine analogues – **TMR-Se**, **10** with a 4-*N,N*-dimethylaminophenyl substituent, **11** with a 4-methoxyphenyl substituent, and **12** with a 3-aminophenyl substitutent – were tested for their phototoxicity toward chemosensitive AUXB1 cells with the data shown in Figure 5. **TMR-Se** and **TMR-S** (Figure 3) displayed nearly identical phototoxicity as did thio-analogue **4** (Figure 3) and its seleno-analogue **11** toward AUXB1 cells. The chalcogen atom had little impact on phototoxicity in spite of its impact on the values of $\phi(^1O_2)$ for **TMR-S**, **TMR-Se**, **4**, and **11** and related compounds. In the absence of light, none of these derivatives showed significant dark toxicity (>90% cell survival) at concentrations up to 1 × 10^{-5} M.

Effects of Verapamil, Oligomycin or Vanadate on Intracellular Accumulation of TMRS in CR1R12 cells. In order to determine whether **TMRS** accumulation would be effected in a dose dependent manner by the addition of the Pgp modulator verapamil, we added various concentrations of verapamil to cultured CR1R12 cells prior to incubation with **TMR-S,** see Materials and Methods. The data displayed in Figure 6 demonstrate that uptake of **TMR-S** is dependent on the verapamil dose added prior to incubation with $1x10^{-5}$ M **TMR-S.** The increase in **TMR-S** uptake was 2 to 4 fold greater in cells exposed to 50 to 200 μM verapamil than in control cells without prior exposure.

In another series of experiments we wanted to determine whether alterations in the energy status of CR1R12 cells, reduction in whole cell ATP levels, would impact the uptake of **TMR-S.** For these studies, we incubated the cell cultures with oligomycin at various concentrations for 2 h prior to the addition of $1x10^{-5}$ M **TMR-S** for 2 h, see Materials and Methods. These data, also depicted in Figure 6, demonstrate that the effect of oligomycin on **TMRS** uptake in CR1R12 cells was dose dependent from 25 to 100 μg/mL oligomycin. No increase in **TMR-S** uptake over that obtained in control cells was observed in the presence of ≤10 μg/mL oligomycin. The data also show that **TMR-S** uptake was 3 to 4 fold higher in the presence of 25 to 100 μg/mL oligomycin compared to control cells not exposed to oligomycin.

Vanadate, which mimics ADP and impairs the ATPase function of Pgp, was added to cultured CR1R12 cells at 20 uM 2 hr prior to **TMR-S** exposure. This resulted in a 3 fold increase in **TMR-S** uptake (data not shown). These data, taken together, demonstrate that accumulation of **TMR-S** in multidrug resistant CR1R12 cells is dependent on Pgp transport function (verapamil modulation), cellular ATP concentration (oligomycin inhibition of FoF1 ATP synthase) and Pgp ATPase function (ADP trapping with vanadate).

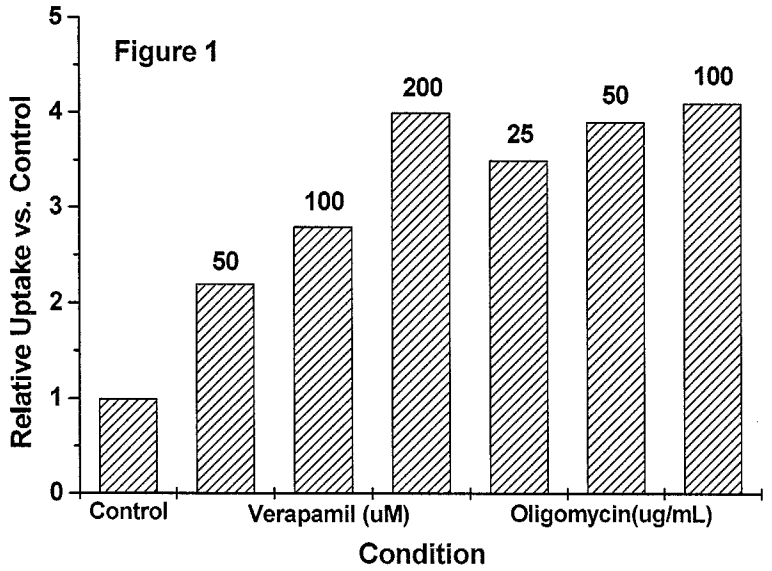


Figure 6. Effects of verapamil or oligomycin on intracellular accumulation of **TMR-S**. Multidrug resistant CR1R12 cells were exposed to 50 or 100 μM verapamil 15 min prior to a 2 hr incubation with **TMR-S** or to 25, 50 or 100 μg/mL oligomycin 2 hr prior to **TMR-S** incubation. Each column represents the mean of at least 3 separate determinations. The data are expressed as the relative increase in **TMR-S** uptake compared to that obtained in control untreated cells.

Photoinactivation of Whole Cell Cytochrome c Oxidase by TMR Analogues. In order to determine whether mitochondria are an intracellular target for **TMR** analogues, we exposed chemosensitive AUXB1 or multidrug resistant CR1R12 cells to **TMRS** or compound **4** or compound **7** for 2 hr followed by 1 hr light exposure, see Materials and Methods for experimental conditions. The data displayed in Figure 7 demonstrate that in AUXB1 cells, all 3 of the **TMR** analogues tested inhibit mitochondrial cytochrome c oxidase by more than 90%. The data also show that following exposure to 50 μM verapamil for 15 min, cytochrome c oxidase in the CR1R12 cells is inhibited to almost the same extent as it was in the AUXB1 cells. In the absence of prior verapamil exposure, however, the inhibition of cytochrome c oxidase in CR1R12 cells by **TMR** analogues had a rank order of **TMR-S**>**4**> **7**. Analysis of this data demonstrated that statistically significant greater cytochrome c oxidase inhibition occurred when data obtained for **TMRS** or **4** were compared with that obtained for **7**, $P<0.05$. These data show that the mitochondria are a target for photosensitization in both sensitive and resistant cell lines.

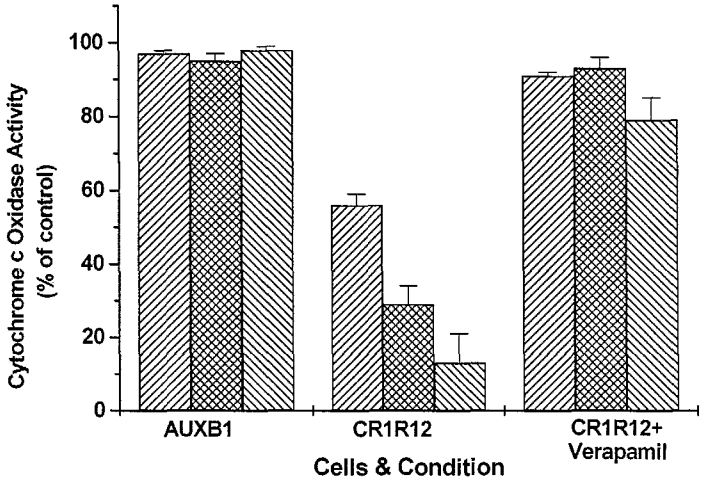


Figure 7. Photoinactivation of whole cell mitochondrial cytochrome c oxidase by **TMR** analogues and light. Cultured chemosensitive AUXB1 or multidrug resistant CR1R12 cells were exposed to **TMRS** (left hash columns), **4** (cross hatch columns) or **7** (right hatch columns) and light and whole cell cytochrome c oxidase activity was determined immediately after light exposure. Details of experimental conditions are presented in the Materials and Methods. Each column represents the mean cytochrome c oxidase activity, expressed as percent of control, obtained from at least 3 separate experiments, error bars are the SEM.

Discussion

A variety of substituents are readily introduced at the 9-position of the 2,7-bis-*N*,*N*-dimethylaminoxanthylium core via the addition of aryl Grignard reagents to the chalcogenoxanthone precursors **1** and **2**. The simplest analogues in the series are **TMR-S** and **TMR-Se** – the thio- and seleno-analogues of **TMR**. From the data in Table 1, the aryl substituents have little impact on wavelengths of absorption and the absorption maxima are dictated by the chalcogen atom. The thio-**TMR** analogues have values of λ_{max} near 570 nm while the seleno-**TMR** analogues have values of λ_{max} near 580 nm. The 9-aryl substituents do impact values of log *P*, the *n*-octanol/water partition coefficient.

The 9-aryl substituents exert a strong influence on photophysical properties. The dominant features of the excited state photophysics are fluorescence, intersystem crossing to the triplet, which can then generate simglet oxygen, and internal conversion. The *N,N*-dimethylamino group markedly increases internal conversion at the expense of fluorescence and intersystem crossing to the triplet (Table 2). The 9-phenyl substituent, 9-4-methylphenyl substituent, 9-4-methoxyphenyl substituent, and 9-3-methoxyphenyl substituent appear to have much smaller contributions from internal conversion. The excited state photophysics for molecules containing these groups is dominated by fluorescence and intersystem crossing for the thio analogues as reflected in values of ϕ_F and $\phi(^1O_2)$ and by intersystem crossing for the seleno analogues as reflected in values of $\phi(^1O_2)$ (Table 2).

The data expressed in Figures 3 and 5 can be extrapolated to give the effective concentrations for 50% cell kill with 5 J cm^{-2} of 350-750-nm light (EC_{50}) for AUXB1 cells and, from Figure 4, the EC_{50} for CR1R12 cells. These values, while approximate, are compiled in Table 3. Based on the EC_{50} values, the rank order of photosensitizer efficiency toward AUXB1 cells is **TMR-S** ≈ **5** > **TMR-Se** > **4** ≈ **11** > **7** > **3** with **6**, **8**, **9**, and **12** showing essentially no phototoxicity at 5×10^{-6} M. The corresponding rank order for values of $\phi(^1O_2)$ is **TMR-Se** > **11** > **TMR-S** ≈ **7** > **5** > **4** > **3**, which suggests that photosensitizer potency is not a simple function of $\phi(^1O_2)$. Toward CR1R12cells, the rank order of potency is **TMR-S** ≈ **5** > **4** > **7** with **3** and **6** showing essentially no phototoxicity at 5×10^{-6} M.

Table 3. Concentrations of photosensitizers **TMR-S**, **TMR-Se**, and **3-12** to give 50% cell kill (EC_{50}) of AUXB1 or CR1R12 cells with 5 J cm^{-2} of 350-750-nm light.

Compd	E	9-Ph-X	$EC_{50} \times 10^6$ M (AUXB1)	$EC_{50} \times 10^6$ M (CR1R12)	$\phi(^1O_2)$, MeOH[a]
TMR-S	S	H	0.1	1.0	0.21[b]
TMR-Se	Se	H	0.2	--	0.87[b]
3	S	4-NMe_2	4.0	>5.0	0.01
4	S	4-OMe	0.3	2.0	0.09
5	S	4-Me	0.1	1.0	0.11
6	S	3-NMe_2	>5.0	>5.0	0.02
7	S	3-OMe	0.7	5.0	0.16
8	S	4-NH_2	>5.0	--	0.02
9	S	3-NH_2	>5.0	--	0.04
10	Se	4-NMe_2	0.7	--	0.02
11	Se	4-OMe	0.3	--	0.60
12	Se	3-NH_2	>5.0	--	0.05

[a] See Table 2 for standard deviation.

For AUXB1 cells, the uptake of **TMR-S**, **4**, and **7** is identical suggesting that the 7-fold differences in values of EC_{50} are due to factors other than uptake and photophysical differences. In the CR1R12 cells, the

phototoxicity and uptake appear to be directly correlated for **TMR-S**, **4**, and **7**, which may relate to the propensity of these materials to be transported by Pgp.

From values of $\phi(^1O_2)$ compiled in Table 2, **TMR-Se** is far more efficient than either **TMR-S** or **5** for the generation of singlet oxygen, yet all three of these molecules have similar potency as photosensitizers. Furthermore, values of log *P* are nearly identical for **TMR-S** and **TMR-Se** (0.07 and -0.09, respectively) and these two molecules are far more hydrophilic than thio derivative **5** (log *P* of 0.81, Table 1). Consequently, phototoxicity does not appear to be a function of lipophilicity. Another pair of interesting comparisons is found in the 4-*N*,*N*-dimethylaminophenyl-substituted derivative **3** and **10**. As shown in Table 2, values of $\phi(^1O_2)$ are low and nearly identical for these two molecules in solution yet seleno analogue **10** has an EC_{50} of 0.7×10^{-6} M toward AUXB1 cells while EC_{50} for **3** is 4×10^{-6} M. Both of these molecules are more lipophilic than **TMR-S** and **TMR-Se**. Clearly, the photosensitizer performance is not simply a function of either log *P* or $\phi(^1O_2)$.

These data, taken together, demonstrate that substituent effects among the various thio- and seleno-tetramethylrosamine analogues impact phototoxicity toward either AUXB1 or CR1R12 cells. In the CR1R12 cells, the addition of verapamil enhances the uptake of the various photosensitizers and the relative phototoxicity of **TMR-S**, **4**, **5**, and **7** in both AUXB1 and CR1R12 cells follow the same trend: **TMR-S** $\approx$ **5** > **4** > **7**.

In an earlier report, we demonstrated that photosensitization of Pgp-expressing CR1R12 cells by **TMR-S** and **TMR-Se** enhanced the uptake of the Pgp transport molecule Calcein AM indicating the these analogues do impact the function of this drug efflux pump[15]. The data presented here demonstrate that substituent effects among the various thio- and seleno-tetramethylrosamine analogues impact their phototoxicity towards either chemosensitive AUXB1 cells or multidrug resistant CR1R12 cells. The substituent effects for the **TMR** analogues were realized in drug resistant CR1R12 cells only when the Pgp modulator verapamil was present indicating that the substituent effects extend to the interaction between the chemical structure of the analogues and binding/transport function of Pgp.

The presence of vanadate, oligomycin, or verapamil increased the level of uptake of **TMR-S**, **4**, or **7** into CR1R12 cells. Presumably, the additives impair the ability of Pgp to remove the rhodamine analogues from the cells. Following treatment with verapamil, the mitochondria are targets for the photosensitizers in the multidrug-resistant CR1R12 cells.

Materials and Methods

General Methods. All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Cell culture media and antibiotics were obtained from Grand Island Biological (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Atlanta, GA).

General Procedure for the Preparation of Chalcogenoxanthylium Dyes. Preparation of 2,7-Bis-*N*,*N*-dimethylamino-9-(4-*N*,*N*-dimethylaminophenyl)thioxanthylium Chloride (3). A mixture of *N*,*N*-dimethyl-4-bromoaniline (0.50 g, 2.5 mmol) and ground magnesium turnings (0.060 g, 2.5 mmol) in 5 mL of anhydrous THF was heated at reflux for 2 h and then cooled to ambient temperature. The resulting solution was then added to a solution of thioxanthenone **1** (0.140 g, 0.47 mmol) in anhydrous THF (5 mL). The reaction mixture was heated at reflux for 1.5 h, cooled to ambient temperature and then to 0 °C. Acetic acid (3.0 mL) was added. Hexafluorophosphoric acid (60 %-by-weight solution in water) was added dropwise until a color change was observed. Ice Water (50 mL) was added and the resulting precipitate was filtered. The precipitate was dissolved in a 50/50 mixture of ethanol/acetonitrile and stirred with Amberlite IRA-400 Chloride exchange resin (0.500 g), for 1 h. The resin was removed by filtration and the ion exchange repeated with fresh resin (2x). The solvent was removed and the resulting solid recrystallized from acetonitrile/ether to yield 0.164 mg (80%) of **3** as a dark purple solid, mp >300 °C ^{1}H NMR (500 MHz, CD_3CN) δ 7.55 (d, 2 H, J = 9.5 Hz), 7.19

(d, 2 H, J = 8.5 Hz), 7.17 (2, 2 H, J = 2.5 Hz), 7.00 (d×d, 2 H, J = 2.5, 9.5 Hz), 6.93 (2, 2 H, J = 9 Hz), 3.21 (s, 12 H), 3.06 (s, 6 H); ^{13}C NMR (75.5 MHz, CD3CN) δ 160.6, 154.4, 152.3, 144.9, 137.5, 132.0, 123.4, 120.2, 116.0, 112.4, 106.4, 40.9, 40.5; HRMS (ES) *m/z* 402.2005 (calcd for $C_{25}H_{28}N_3S^+$: 402.1998); λ_{max} (CH_2Cl_2) 564 nm (ε = 1.00 × 10^5 $M^{-1}cm^{-1}$), λ_{max} (CH3OH) 565 nm (ε = 1.05 × 10^5 $M^{-1}cm^{-1}$). **Anal.** Calcd for $C_{25}H_{28}N_3SCl$: C, 68.55; H, 6.44; N, 9.59. Found: C, 68.57; H, 6.31; N, 9.61.

Preparation of 2,7-Bis-*N,N*-dimethylamino-9-(4-methoxyphenyl)thioxanthylium Chloride (4). 4-Bromoanisole (0.935 g, 5.0 mmol), ground magnesium turnings (0.12 g, 5.0 mmol), and thioxanthen-9-one **1** (0.10 g, 0.34 mmol) were treated as described. Following the final ion exchange, the filtrate was concentrated and the crude chloride salt was recrystallized from acetonitrile/ether to give 0.10 g (70%) of **4** as a dark purple solid, mp >300 °C: ^{1}H NMR (400 MHz, CD_3CN) δ 7.42 (d, 2 H, J = 9.6 Hz), 7.27 (d, 2 H, J = 8.8 Hz), 7.20 (d, 2 H, J = 2.4 Hz), 7.17 (d, 2 H, J = 8.8 Hz), 6.99 (d×d, 2 H, J = 2.4, 9.6 Hz), 3.91 (s, 3 H), 3.21 (s, 12 H); ^{13}C NMR (75.5 MHz, CD_3CN) δ 161.6, 161.3, 154.6, 145.1, 137.3, 131.8, 128.7, 120.2, 116.3, 115.1, 106.6, 56.3, 41.0; HRMS (ES) *m/z* 389.1684 (calcd for $C_{24}H_{25}N_2OS^+$: 389.1682); λ_{max} (CH_2Cl_2) 569 nm (ε = 1.10 × 10^4 $M^{-1}cm^{-1}$), λ_{max} (CH_3OH) 569 nm (ε = 9.8 × 10^4 $M^{-1}cm^{-1}$). **Anal.** Calcd for $C_{24}H_{25}N_2OSCl$: C, 67.83; H, 5.93; N, 6.59. Found: C, 67.68; H, 6.04; N, 6.47.

Preparation of 2,7-Bis-*N,N*-dimethylamino-9-(4-methylphenyl)thioxanthylium Chloride (5). *p*-Tolylmagnesiumbromide (1.34 mL, 1.3 mmol, 1 M solution in ether) and thioxanthen-9-one **1** (0.10 g, 0.34 mmol) were treated as described. Following the final ion exchange, the crude chloride salt was recrystallized from acetonitrile/ether to give 0.136 g (98%) of **5** as a dark purple solid, mp >300 °C: ^{1}H NMR (400 MHz, CD_3CN) δ 7.45 (d, 2 H, J = 8.0 Hz), 7.36 (d, 2 H, J = 10 Hz), 7.23 (d, 2 H, J = 8.0 Hz), 7.20 (d, 2 H, J = 2.8 Hz), 6.98 (d×d, 2 H, J = 2.8, 10 Hz), 3.21 (s, 12 H), 2.49 (s, 3 H); ^{13}C NMR (125.5 MHz, CD_3CN) δ 161.3, 154.4, 145.0, 140.6, 137.1, 133.8, 130.2, 130.1, 119.9, 116.3, 106.5, 41.0, 21.4; HRMS (ES) *m/z* 373.1736 (calcd for $C_{24}H_{25}N_2S^+$: 373.1738); λ_{max} (CH_2Cl_2) 569 nm, (ε = 13.1 × 10^4 $M^{-1}cm^{-1}$); λ_{max} (CH_3OH) 570 nm (ε = 9.5 × 10^4 $M^{-1}cm^{-1}$). **Anal.** Calcd for $C_{24}H_{25}N_2SCl$: C, 67.83; H, 5.93; N, 6.59. Found: C, 67.84; H, 5.86; N, 6.62.

Preparation of 2,7-Bis-*N,N*-dimethylamino-9-(3-*N,N*-dimethylaminophenyl)thioxanthylium Chloride (6). *N,N*-Dimethyl-3-bromoaniline (0.50 g, 2.5 mmol), ground magnesium turnings (0.060 g, 2.5 mmol), and thioxanthen-9-one **1** (0.14 g, 0.47 mmol) were treated as described. The crude chloride salt was recrystallized from acetonitrile and a small amount of ether to give 0.154 g (75 %) of **10** as a dark green solid, mp >300°C: ^{1}H NMR (500 MHz, CD_3OD) δ 7.62 (t, 1 H, J = 8.0 Hz), 7.52 (d, 2 H, J = 9.5 Hz), 7.38-7.32 (m, 3 H), 7.14 (dxd, 2 H, J = 2.5, 9.5 Hz), 7.08 (br s, 1 H), 6.98 (br s, 1 H), 4.91 (s, 12 H), 3.15 (s, 6 H); ^{13}C NMR (75.5 MHz, 50/50 CD_3OD/CD_2Cl_2) δ 160.8, 154.5, 148.7, 145.3, 137.8, 137.2, 130.7, 123.0, 119.8, 116.9 (2C), 116.1, 106.2, 42.9, 40.8; HRMS (ES) *m/z* 402.1996 (calcd for $C_{25}H_{28}N_3S^+$: 402.1998); λ_{max} (CH_2Cl_2) 573 nm (ε = 1.09 x 10^5 $M^{-1}cm^{-1}$), λ_{max} (CH_3OH) 573 nm, (ε = 7.3 x 10^4 $M^{-1}cm^{-1}$). **Anal.** Calcd for $C_{25}H_{28}N_3SCl$: C, 68.55; H, 6.44; N, 9.59. Found: C, 68.19; H, 6.51; N, 9.41.

Preparation of 2,7-Bis-*N,N*-dimethylamino-9-(3-methoxyphenyl)thioxanthylium Chloride (7). 3-Bromoanisole (0.935 g, 5.0 mmol), ground magnesium turnings (0.12 g, 5.0 mmol), and thioxanthen-9-one **1** (0.10 g, 0.34 mmol) were treated as described. Following the final ion exchange, the crude chloride salt was recrystallized from acetonitrile/ether to give 0.070 g (40%) of 7 as a dark purple solid, mp >300 °C: ^{1}H NMR (400 MHz, CD_3CN) δ 7.54 (t, 1 H, J = 8.2 Hz), 7.39 (d, 2 H, J = 9.6 Hz), 7.22 (d, 2 H, J = 2.4 Hz), 7.18 (d×d×d, 1 H, J = 1.0, 2.6, 8.2 Hz), 6.99 (d×d, 2 H, J = 9.6, 2.4 Hz), 6.93-6.91 (m, 2 H), 3.82 (s, 3 H), 3.22 (s, 12 H); ^{13}C NMR (75.5 MHz, CD_3CN) δ160.7, 160.5, 154.5, 145.1, 138.1, 137.1, 130.9, 122.4, 119.6, 116.4, 116.0, 115.6, 106.7, 56.2, 41.1; HRMS (ES) *m/z* 389.1684 (calcd for $C_{24}H_{25}N_2OS^+$: 389.1682); λ_{max} (CH_2Cl_2) 572 nm (ε = 9.6 × 10^4 $M^{-1}cm^{-1}$), λ_{max} (CH_3OH) 571 nm (ε = 8.7 × 10^4 $M^{-1}cm^{-1}$). **Anal.** Calcd for $C_{24}H_{25}N_2OSCl$: C, 67.83; H, 5.93; N, 6.59. Found: C, 67.76; H, 5.91; N, 6.71.

Preparation of 2,7-Bis-*N,N*-dimethylamino-9-(4-aminophenyl)thioxanthylium Chloride (8). 1-(4-Bromophenyl)-2,2,5,5-tetraethyl-1,2,5-azadisilolidine (0.50g, 1.6 mmol), magnesium turnings (0.040 g, 1.7 mmol), and thioxanthen-9-one **1** (0.119 g, 0.40 mmol) were treated as described. Following the final ion exchange, the the crude chloride salt was recrystallized from CH_3CN/ether to give 0.095 g (58 %) of a dark green solid, mp °C: ^{1}H NMR (500 MHz, CD_3OD) δ 7.68 (d, 2 H, J = 10 Hz), 7.30 (d, 2 H, J = 2.5 Hz), 7.12 (d×d, 2 H, J = 2.5, 10 Hz), 7.09 (d, 2 H, J = 8 Hz), 6.91 (d, 2 H, J = 8 Hz), 3.29 (s, 12 H); ^{13}C NMR (75.5 MHz, CD_2Cl_2) δ 153.80, 144.74, 137.44, 131.46, 120.07, 115.29, 114.71, 105.66, 40.84; λ_{max} (CH_3OH) 565 nm (ε = 7.63×10^4 M^{-1} cm^{-1}); HRMS (ESI) *m/z* 374.1697 (Calcd for $C_{23}H_{24}N_3S^+$: 374.1685). **Anal.** Calcd for $C_{23}H_{24}N_3SCl$: C, 67.38; H, 5.90; N, 10.25. Found: C, 67.22; H, 5.95; N, 10.26.

Preparation of 2,7-Bis-*N,N*-dimethylamino-9-(3-aminophenyl)thioxanthylium Chloride (10). 1-(3-Bromophenyl)-2,2,5,5-tetraethyl-1,2,5-azadisilolidine (0.50g, 1.6 mmol), magnesium turnings (0.040 g, 1.7 mmol), and thioxanthen-9-one **1** (0.119 g, 0.40 mmol) were treated as described. Following the final ion exchange, the crude chloride was recrystallized from acetonitrile and a small amount of diethyl ether to give 0.088g (54 %) of the product as a dark green solid, mp °C: ^{1}H NMR (500 MHz, CD_3OD) δ 7.64 (t, 1 H, J = 7.5, 8.5 Hz), 7.45 (d, 2 H, J = 9.5 Hz), 7.38 (m, 1 H), 7.36 (d, 2 H, J = 2.5 Hz), 7.15 (m, 2 H), 7.12 (dxd, 2 H, J = 2.5, 9.5 Hz), 3.31 (s, 12 H); ^{13}C NMR (125 MHz, CD_3OD, 45 °C) δ 160.9, 155.3, 145.8, 138.5, 137.5, 137.4, 131.2, 131.1, 124.4, 120.3, 120.0, 116.7, 106.8, 40.7; λ_{max} (MeOH) 571 nm (ε = 3.04×10^4 M^{-1} cm^{-1}); HRMS (ESI) *m/z* 374.1695 (Calcd for $C_{23}H_{24}N_3S^+$: 374.1685). **Anal.** Calcd for $C_{23}H_{24}N_3SCl$: C, 67.38; H, 5.90; N, 10.25. Found: C, 67.05; H, 6.01; N, 10.04.

Preparation of 2,7-Bis-*N,N*-dimethylamino-9-(4-*N,N*-dimethylaminophenyl)selenoxanthylium Chloride (10). *N,N*-Dimethyl-4-bromoaniline (0.23g, 1.2 mmol), ground magnesium turnings (0.030 g, 1.2 mmol), and selenoxanthen-9-one **2** (0.10 g, 0.29 mmol) were treated as described. Following the final ion exchange, the crude chloride salt was recrystallized from CH_3CN and a small amount of ether to give 0.089 g (69 %) of **10** as a dark green solid, mp 251-252°C: ^{1}H NMR (500 MHz, CD_2Cl_2) δ 7.68 (d, 2 H, J = 9.8 Hz), 7.24 (d, 2 H, J = 2.4 Hz), 7.14 (d, 2 H, J = 8.5 Hz), 6.87 (d, 2 H, J = 8.5 Hz), 6.84 (d×d, 2 H, J = 2.4, 9.8 Hz), 3.24 (s, 12 H), 3.09 (s, 6 H); ^{13}C NMR (125 MHz, CD_2Cl_2) δ 153.5, 144.4, 136.5, 135.4, 129.5, 129.2, 128.7, 119.2, 115.1, 114.2, 105.4, 40.5; λ_{max} (CH_2Cl_2) 576 nm (ε = 5.6×10^4 M^{-1} cm^{-1}) 580 nm (sh, ε = 5.7×10^3 M^{-1} cm^{-1}); HRMS (ESI) *m/z* 450.1440 (Calcd for $C_{25}H_{28}N_3{}^{80}Se$: 450.1443). **Anal.** Calcd for $C_{25}H_{28}N_3SeCl$: C, 61.92; H, 5.82; N, 8.67. Found: C, 61.83; H, 5.95; N, 8.47.

Preparation of 2,7-Bis-*N,N*-dimethylamino-9-(4-methoxyphenyl)selenoxanthylium Chloride (11). 4-Bromoanisole (1.62 g, 17.0 mmol), ground magnesium turnings (0.22 g, 8.0 mmol), and selenoxanthen-9-one **2** (0.15 g, 0.43 mmol) were treated as described. Following the final ion exchange, the crude chloride salt was recrystallized from acetonitrile/ether to give 0.12 g (70%) of **10** as a black solid, mp 245-246 °C: ^{1}H NMR (500 MHz, CD_2Cl_2) δ 7.51 (d, 2 H, J = 9.8 Hz), 7.45 (d, 2 H, J = 2.7 Hz), 7.21 (d×d, 2 H, J = 1.8, 6.7 Hz), 7.11 (d×d, 2 H, J = 1.8, 6.7 Hz), 6.83 (d×d, 2 H, J = 2.7, 9.8 Hz), 3.26 (s, 12 H), 2.92 (s, 3 H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 157.4, 148.9, 134.3, 130.9, 130.0, 128.6, 128.1, 126.5, 113.5 , 112.9, 111.6, 54.8, 40.7; λ_{max} (CH_2Cl_2) 576 nm (ε = 5.6×10^4 M^{-1} cm^{-1}), 578 nm, (sh, ε = 1.7×10^3 M^{-1} cm^{-1}); HRMS (ESI) *m/z* 437.1133 (Calcd for $C_{24}H_{25}N_2O\,{}^{80}Se^+$: 437.1127). **Anal.** Calcd for: C, 61.09; H, 5.34; N, 5.94. Found: C, 60.94; H, 5.48; N, 5.95.

Preparation of 2,7-Bis-*N,N*-dimethylamino-9-(3-aminophenyl)selenoxanthylium Chloride (12). 1-(3-Bromophenyl)-2,2,5,5-tetraethyl-1,2,5-azadisilolidine (0.50g, 1.6 mmol), magnesium turnings (0.040 g, 1.7 mmol), and selenoxanthen-9-one **2** (0.14 g, 0.40 mmol) were treated as described. Following the final ion exchange, the crude chloride was recrystallized from CH_3CN and a small amount of diethyl ether to give 0.070g (17 %) of the product as a dark green solid, mp 291-292 °C: ^{1}H NMR (500 MHz, CD_2Cl_2) δ 7.66 (d, 2 H, J = 9.8 Hz), 7.48 (d, 2 H, J = 2.1 Hz), 7.35 (t, 1 H, J = 7.5 Hz), 6.92 (d×d, 1 H, J = 1.5, 7.4 Hz), 6.87 (d×d, 2 H, J= 2.1, 9.8 Hz), 6.74 (d×d, 1 H, J = 1.5Hz), 6.62 (d, 1 H, J = 7.6Hz) 4.50 (br s, 2 H), 3.29 (s, 12 H); ^{13}C NMR (300 MHz, CD_3OD) δ 154.7, 147.2, 139.7, 139.6, 130.7, 122.0, 120.7, 118.6, 118.4 , 116.1, 110.0, 40.7; λ_{max}

(CH_3OH) 582 nm ($\varepsilon = 5.6 \times 10^4$ M^{-1} cm^{-1}); λ_{max} (CH_2Cl_2) = 582 nm, ($\varepsilon = 3.7 \times 10^3$ M^{-1} cm^{-1}); HRMS (ESI) *m/z* 422.1142 (Calcd for $C_{23}H_{24}N_3{}^{80}Se^+$: 422.1130). **Anal.** Calc for $C_{23}H_{24}N_3SeCl$: C, 60.46; H, 5.29; N, 9.20. Found: C, 60.22; H, 5.51; N, 8.99.

Preparation of 1-(4-Bromophenyl)-2,2,5,5-tetramethyl-1,2,5-azadisilolidine.[18] 4-Bromoanaline (1.00 g, 5.81 mmol), triethylamine (1.62 ml, 11.6 mmol) and 4-(dimethylamino)pyridine (0.070 g. 0.58 mmol) were dissolved in CH_2Cl_2 (20 ml) at ambient temperature. 1 ,2-Bis(chlorodimethylsilyl)ethane (1.25 g, 5.81 mmol) in 5 mL of CH_2Cl_2 was added slowly and the resulting mixture was stirred for 3 h. Upon completion, hexanes (30 ml) were added to the reaction mixture precipitating triethylammonium chloride, which was removed by filtration. The resulting pale yellow oil was purified by column chromatography on basic alumina, eluted with petroleum ether/ethyl acetate (4/1) to give the stabase-protected aniline in 1.66 g (91 %) isolated yield: ^{1}H NMR ($CDCl_3$) δ 7.34 (AA'BB', 2 H, J = 8.5 Hz), 6.84 (AA'BB', 2 H, J = 8.5 Hz), 0.91 (s, 4 H), 0.25 (s, 12 H).

Preparation of 1-(3-Bromophenyl)-2,2,5,5-tetramethyl-1,2,5-azadisilolidine.[18] 3-Bromoanaline (1.00 g, 5.81 mmol), triethylamine (1.62 ml, 11.6 mmol), 4-(dimethylamino)pyridine (0.070 g. 0.58 mmol), and 1,2-bis(chlorodimethylsilyl)ethane (1.25 g, 5.81 mmol) were treated as described to give 1.66 g (93 %) of a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.34 (AA'BB', 2 H, J = 8.5 Hz), 6.81 (AA'BB', 2 H, J = 8.5 Hz), 0.91 (s, 4 H), 0.25 (s, 12 H).

Preparation of Bis-2,7-*N*,*N*-dimethylaminothioxanthen-9-one (1). Phosphorus oxychloride (2.7 mL, 29 mmol) and arylthiobenzamide **13** (1.08 g, 2.9 mmol) were heated at reflux in 50 mL of acetonitrile for 1.5 h and the reaction mixture was then poured into a mixture of 75 mL of 1 M NaOH and 75 g of ice. The resulting mixture was stirred for 1 h and the products were extracted with CH_2Cl_2 (3 × 50 mL). The combined organic extracts were washed with brine, dried over $MgSO_4$, and concentrated. The crude product was purified via recrystallization from CH_2Cl_2/hexanes to give 0.65 g (75%) of **1** as a yellow crystalline solid, mp 261-262 °C (lit.[14] mp: 260- 261 °C): ^{1}H NMR (500 MHz, CD_2Cl_2) δ 8.42 (d, 2 H, J = 9.2 Hz), 6.81 (dxd, 2 H, J = 2.4, 9.2 Hz), 6.74 (d, 2 H, J = 2.4 Hz), 3.11 (s, 12 H), ^{13}C NMR (125 MHz, CD_2Cl_2) δ 177.2, 151.7, 138.6, 130.3, 118.5, 110.9, 104.8, 39.6; IR (KBr) 1589 cm^{-1}; λ_{max} (EtOH) 377 nm.

Preparation of Bis-2,7-dimethylaminoselenoxanthen-9-one (2). Phosphorus oxychloride (2.4 mL, 26 mmol) and benzamide **14** (1.09 g, 2.6 mmol) were heated at reflux in 30 mL of acetonitrile for 1.5 h. Workup as described above gave 0.66 g (73%) of **2**, mp 224-225 °C (lit.[16] mp: 224-225 °C): ^{1}H NMR [500MHz, CD_2Cl_2] δ 8.38 (d, 2 H, J = 8.9 Hz), 6.80 (d×d, 2 H, J = 8.9, 1.2 Hz), 6.75 (d, 2 H, J = 1.2 Hz), 3.11 (s, 12 H); ^{13}C NMR (CD_2Cl_2) δ 179.1, 151.6, 136.1, 131.6, 119.9, 111.0, 107.5, 39.5; IR (KBr) 1592 cm^{-1}; λmax (EtOH) 388 nm.

Quantum yield determination for the generation of singlet oxygen. The quantum yields for the generation of singlet oxygen for **TMR-S**, **TMR-Se**, and **3-12** were measured by direct methods in methanol or chloroform.[19] A SPEX 270M spectrometer (Jobin Yvon) equipped with InGaAs photodetector (Electro-Optical Systems, Inc., USA) was used for recording singlet–oxygen emission spectra. A diode-pumped solid-state laser (Millenia X, Spectra Physics) at 532 nm was the excitation source. The sample solution in a quartz cuvette was placed directly in front of the entrance slit of the spectrometer and the emission signal was collected at 90° relative to the exciting laser beam. An additional longpass filter (850LP) was used to attenuate the excitation laser and the fluorescence from the photosensitizer.

Fluorescence Quantum Yields. Fluorescence quantum yields (ϕ_F) were measured using techniques and equipment that we have previously described.[20]

Cells and culture conditions. Cultured cells used in this study were the Chinese hamster ovary parental cell line AUXB1,[21] a chemo-sensitive cell line which has a very low Pgp content and the multidrug resistant subline, CR1R12, which highly constituitively expresses Pgp. Multidrug resistant CR1R12 cells were established from the CH^RC5 cell line[22] by sequential culturing in increasing concentrations of colchicine with 5μg/mL being the final concentration used. Cell lines were maintained in passage culture on 60mm diameter

polystyrene dishes (Corning Costar, Corning, NY) in 3.0mL Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin G, 50 μg/ml streptomycin and 1.0μg/mL Fungizone® (complete medium). Only cells from passages 1-10 were used for experiments. A stock of cells, passages 1-4, were maintained at -86 °C to initiate experimental cultures. Cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere (Forma Scientific, Marietta, OH). Passage was accomplished by removing the culture medium, then adding a 1.0 mL solution containing 0.25% trypsin, incubating at 37 °C for 2 to 5 minutes to remove cells from the surface and seeding new culture dishes with an appropriate number of cells in 3.0 mL of complete medium. Cell counts were performed using either a hemocytometer or a particle counter (Model ZM, Coulter Electronics, Hialeah, FL).

Determination of intracellular dye content. Cell lines, AUXB1 or CR1R12, were seeded on 96-well plates in 200μL/well complete medium at 1-4x10^4 cells/well. Twenty four hours after seeding, verapamil at 50 μM was added to selected wells in complete medium and cultures were incubated in the dark at 37 °C for 15 min. Dyes were then added to the cultures at various concentrations in complete medium. Cells were incubated in the dark at 37 °C for 2 h with or without verapamil in the presence of selected dyes. The medium was then removed and the monolayers washed once with 200 μL 0.9% NaCl and 200 μL 0.9% NaCl were added. The fluorescence of the intracellular dye was determined using a multi-well fluorescence plate reader (Gemini, Molecular Devices, Palo Alto, CA). The excitation/emission wavelengths were set appropriately for each dye. Intracellular dye content was determined by comparing the magnitude of the fluorescence signal measured in each well to the fluorescence signal emitted from known concentrations of dye and calculating the femtomoles of intracellular dye/cell from those values.

Photoradiation of Cell Cultures. Cell lines, AUXB1 or CR1R12, were seeded on 96-well plates in 200 μL/well complete medium at 1-4×10^4 cells/well. Following treatment with 1 × 10^{-5} M verapamil or not, **TMR** analogues were then added directly to the cell culture medium at various concentrations and incubated for 2 h in the dark as above. The medium was then removed and 200 μL α-MEM minus FBS and phenol red (clear medium) were added to each well. One plate, with the lid removed, was then exposed to 350-750-nm light delivered at 1.4mW cm^{-2} for 1 h (5.0 J cm^{-2}) from a filtered tungsten/halogen source while a parallel plate was kept in the dark during the irradiation period. Immediately following irradiation the clear medium was replaced with complete medium and the monolayers were incubated for an additional 24-h period. Subsequently, cells were trypsinized and the MTT assay was used to determine cell viability. Data are expressed as percent cell viability compared to control cells which had been exposed to neither dye nor light.

Whole Cell Cytochrome c Oxidase Measurements. To determine whether dyes photosensitized mitochondrial cytochrome *c* oxidase in cultured cells, AUXB1 or CR1R12 cells were plated on 12-well culture plates using an initial cell seeding of 1.0×10^5 cells/well in 1.0 mL complete medium. Cells were incubated for 24 h at 37 °C in a 5% CO_2 humidified atmosphere as described above. Following the 24-h incubation period, **TMR** analogues were added at 1 × 10^{-6} M final concentration. Control cells, no added dye, were maintained under the same conditions. Cells were incubated for 2 h in the dark, the medium was removed and replaced with 1.0 mL clear medium, medium without pheno red. Monolayers were then exposed to 350-750 nm light delivered at 1.4mW/cm^2 for 1 hr (5.0J/cm^2) from a filtered tungsten/halogen source as described earlier (ref). Following irradiation of the cultures, the medium was replaced with 1.0 mL complete medium and irradiated plates and plates that remained in the dark were incubated for 24 h at 37 °C as above. Subsequently, 0.1 mL trypsin was added to each well to detach the cells. Cell suspensions from three similarly treated wells were then combined and transferred to 1.0–mL microcentrifuge tubes and centrifuged at 8000 × g for 3 min. The supernatant was aspirated and cell pellets were immediately frozen and stored at –86 °C. Cytochrome c oxidase activity was determined on 1 to 1.5 × 10^6 cells that had been thawed in 0.1 mL 0.9%NaCl and sonicated for 10 s on ice using a Bronson sonicator (Model 185, Brinkmann Ind.) at a setting of 2. Measurement of cytochrome *c* oxidase was performed according to a method described earlier (ref). Data are expressed as percent of control

cytochrome *c* oxidase activity, mols of cytochrome *c* oxidized/min/cell, which was determined from cells not exposed to dye or light. Cytochrome *c* oxidase activity was also determined in cells exposed to dye alone or light alone, drug and light controls respectively.

Intracellular Accumulation of TMRS in the Presence of Oligomycin or Vanadate. CR1R12 cells were seeded on 96-well plates in 200μL/well complete medium at 1-4x10^4 cells/well. Twenty four hours after seeding, oligomycin at 25, 50 or 100 ug/mL or vanadate at 20 uM were added for 2 hr at 37^{0}C in the dark. For comparison, in another set of wells, 50 uM verapamil was added for 15 min prior to the addition of **TMRS**. Following the incubation periods with either oligomycin or verapamil, **TMRS** was added at 1x10^{-5}M for 2 hr in the dark. The medium was then removed and the monolayers washed once with 200μL 0.9% NaCl and 200μL 0.9% NaCl were added. The fluorescence of the intracellular dye was then determined using a multi-well fluorescence plate reader (Gemini, Molecular Devices, Palo Alto, CA). Dye uptake in oligomycin or verapamil treated CR1R12 cells was compared to uptake in control untreated cells, data are expressed as relative **TMRS** uptake vs that obtained for control cells.

Key Research Accomplishments

- Publications in Print: Gibson, S. L.; Holt, J. J.; Ye, M.; Donnelly, D. J.; Ohulchanskyy, T. Y.; You, Y.; Detty, M. R. "Structure-activity Studies of Uptake and Phototoxicity with Heavy-chalcogen Analogues of Tetramethylrosamine *in vitro* in Chemosensitive and Multidrug-resistant Cells," *Biorg. Med. Chem.* **2005**, *13*, 6394-6403.
- Manuscripts in Preparation: Gibson, S. L.; Tombline, G.; Donelly, D. J.; Holt, J. J.; Senior, A. E.; Detty, M. R. "Structure-activity Studies of Photoinactivation of P-glycoprotein in Multidrug-resistant CR1R12 Cells and in Isolated P-glycoprotein," to be submitted to *J. Med. Chem.*, December, 2005.

Reportable Outcomes

- National Institutes of Health R01 Grant Application submitted using preliminary data generated by W81XWH-04-1-0708
- Department of Defense Predoctoral Fellowship Application submitted using preliminary data generated by W81XWH-04-1-0708
- Four presentations concerning photoinhibition of P-glycoprotein in cultured cells and isolated protein (Portland State University, Portland, OR; Geneseo College, Geneseo, NY; Rochester Section of the American Chemical Society Dinner Talk, Rochester, NY; University at Albany, Albany, NY)

Conclusions

The work completed under this award demonstrates that substituent effects among the various thio- and seleno-trhodamine analogues impact their phototoxicity towards either chemosensitive AUXB1 cells or multidrug resistant CR1R12 cells. The substituent effects for the rhodamine analogues were realized in drug resistant CR1R12 cells only when the Pgp modulator verapamil was present indicating that the substituent effects extend to the interaction between the chemical structure of the analogues and binding/transport function of Pgp. The presence of vanadate, oligomycin, or verapamil increased the level of uptake of **TMR-S**, **4**, or **7** into CR1R12 cells. Presumably, the additives impair the ability of Pgp to remove the rhodamine analogues from the cells. Following treatment with verapamil, the mitochondria are targets for the photosensitizers in the multidrug-resistant CR1R12 cells.

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Appendix

A. Bibliography (Manuscripts from W81XWH-04-1-0708)

Gibson, S. L.; Holt, J. J.; Ye, M.; Donnelly, D. J.; Ohulchanskyy, T. Y.; You, Y.; Detty, M. R. "Structure-activity Studies of Uptake and Phototoxicity with Heavy-chalcogen Analogues of Tetramethylrosamine *in vitro* in Chemosensitive and Multidrug-resistant Cells," *Biorg. Med. Chem.* **2005**, *13*, 6394-6403

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- Principal Investigator: Michael R. Detty
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- Graduate Student: Jason Holt

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Structure–activity studies of uptake and phototoxicity with heavy-chalcogen analogues of tetramethylrosamine in vitro in chemosensitive and multidrug-resistant cells

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Abstract—Several thio and seleno analogues of tetramethylrosamine (**TMR**) were prepared. Thio derivatives of **TMR** have absorption maxima near 570 nm, while seleno derivatives of **TMR** have absorption maxima near 580 nm. The 3- or 4-*N*,*N*-dimethylaminophenyl substituent in the 9-position greatly increases internal conversion, which lowers quantum yields for fluorescence and the generation of singlet oxygen. Thio and seleno analogues of **TMR** are effective photosensitizers against chemosensitive AUXB1 cells in vitro and against multidrug-resistant CR1R12 cells in vitro, which have been treated with verapamil. The CR1R12 cells accumulated significantly lower concentrations of the photosensitizers relative to the AUXB1 cells presumably due to the expression of P-glycoprotein (Pgp) in the CR1R12 cells. Following treatment with 5×10^{-5} M verapamil, the uptake in CR1R12 cells of several fluorescent thio analogues of **TMR** is comparable to that observed for the chemosensitive AUXB1 cells.

1. Introduction

Multidrug resistance (MDR) has been identified as a major impediment to successful chemotherapy for a variety of cancers.[1] Multidrug resistance is attributed to the action of a class of membrane proteins termed the ATP-binding cassette (ABC) transporters.[1–3] These proteins, P-glycoprotein (Pgp), the multidrug resistance protein (MRP) series, breast cancer resistance protein (BCRP), and lung-resistance-related protein (LRP), are involved in the transport of substrates that span a wide range of chemically diverse and structurally disparate compounds including a majority of the commonly prescribed chemotherapeutic agents.[4] The ABC transport proteins possess similarities such as their dependence on the energy derived from ATP hydrolysis to transport substrates,[5] the presence of units of six transmembrane domains (TMDs) which are poorly conserved among the different proteins, and the presence of nucleotide-binding domains (NBDs) which are highly conserved.[1–3] The majority of the ABC transporters are responsible for the transmembrane movement of ions and compounds to maintain levels necessary for cell viability. The drug resistance proteins in this class, of which P-glycoprotein is reportedly the most prevalent,[6] have the unique ability to pump a broad array of chemotherapeutics actively out of the cells, dramatically reducing drug concentration and thus their therapeutic effectiveness.[1,6] Interestingly, expression of Pgp and its recognition of various substrates can be induced by exposure to a single compound or therapeutic agent.[7] Due to the diverse nature of the molecules transported by Pgp, its function as an efflux pump is of major concern for clinicians and under intense investigation by numerous groups worldwide.[8]

Attempts have been made to alleviate the function of Pgp using modulators such as verapamil, quinidine,

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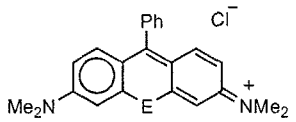


TMR, E = O
TMR-S, E = S
TMR-Se, E = Se

Chart 1. Structure of tetramethylrosamine (**TMR**) and thio- (**TMR-S**) and seleno-analogues (**TMR-Se**).

cyclosporin A, PSC833, and others.[1,9] These modulators bind to the drug-transport-binding site(s) on the protein and impede the binding and subsequent efflux of agent(s) of interest, thus allowing their accumulation.

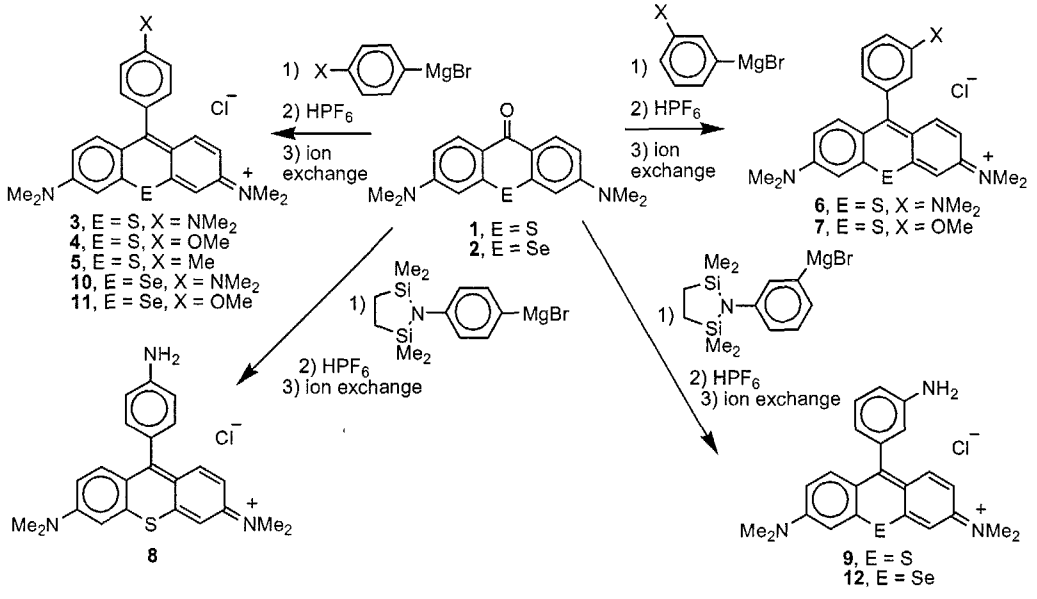
Studies of the efflux function of Pgp have traditionally used Pgp transport molecules that are either intrinsically fluorescent or tagged with a fluorescent marker.[10,11] Assays employing such compounds usually monitor the efflux of a specific molecule, such as Rhodamine 123, from cells by measuring a decrease in intracellular fluorescence.[11] In addition, the quenching of tetramethylrosamine (**TMR**) fluorescence has been used to measure transport by Pgp in isolated membrane vesicles.[12]

We have been studying heavy-chalcogen analogues of the rhodamine dyes and **TMR** (Chart 1) for their utility as phototoxic agents for malignant cells and tissues.[13,14] These compounds have an affinity for mitochondrial localization in cancer cells due to their delocalized positive charge. The insertion of the heavy-chalcogen atoms sulfur, selenium, or tellurium into these compounds, replacing oxygen in the rhodamines and **TMR**, allows for higher triplet yields and, consequently, higher quantum yields for the generation of singlet oxygen ($^1O_2$). The generation of $^1O_2$ results in damage to intracellular structures and functions ultimately leading to cell death. The use of photosensitizers in combination with Pgp modulators can reduce toxicity toward normal tissues during treatment of multidrug-resistant cancers since the activating light for the photosensitizer can be delivered specifically to the areas of treatment. We extend our initial studies of thiotetramethylrosamine (**TMR-S**) and selenotetramethylrosamine (**TMR-Se**) as photosensitizers for both chemosensitive and multidrug-resistant cells, as transport molecules for Pgp, and as inhibitors of Pgp-mediated multidrug resistance[15] to include a series of structurally related derivatives. In this report, we compare the uptake and phototoxicity of a series of thio and selenotetramethylrosamine analogues in chemosensitive AUXB1 cells and in multidrug-resistant, highly Pgp-expressing CR1R12 cells in which Pgp function has been inhibited by the addition of verapamil. In the presence of verapamil, several of the thiotetramethylrosamine derivatives are effective photosensitizers for multidrug-resistant CR1R12 cells.

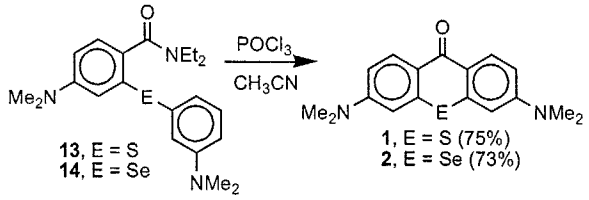
2. Results

2.1. Synthesis and properties of dyes 3–12

The synthetic approach to the synthesis of **TMR-S** and **TMR-Se** involves the addition of phenylmagnesium bromide to chalcogenoxanthen-9-ones **1** and **2**, respectively, followed by acid-induced dehydration and ion exchange.[14] To investigate structure–activity relationships within a series of related molecules, the chalcogenoxanthylium dyes **3**–**12** of Scheme 1 were prepared via a similar approach. The Grignard reagents prepared from 3-bromo or 4-bromo derivatives of various substituted benzenes were added to the chalcogenoxanthen-9-ones



Scheme 1. Synthesis of thio- and seleno-analogues of **TMR**.



Scheme 2. Synthesis of chalcogenoxanthen-9-one **1** and **2**.

Table 1. Absorption maxima (λ_{max}) and molar extinction coefficients (ε) in MeOH and *n*-octanol/water partition coefficients (log *P*) for **TMR-S**, **TMR-Se**, and **3–12**

Compound	E	9-Ph-X	MeOH		log P^a
			λ_{max} (nm)	ε ($M^{-1}cm^{-1}$)	
TMR-S	S	H	571	50,100	0.07 ± 0.02
TMR-Se	Se	H	582	69,200	−0.09 ± 0.09
3	S	4-NMe_2	573	72,400	0.94 ± 0.01
4	S	4-OMe	569	97,700	−0.09 ± 0.03
5	S	4-Me	570	95,400	0.81 ± 0.04
6	S	3-NMe_2	573	72,400	1.13 ± 0.02
7	S	3-OMe	571	87,100	0.14 ± 0.01
8	S	4-NH_2	565	75,800	0.20 ± 0.02
9	S	3-NH_2	571	51,000	0.05 ± 0.03
10	Se	4-NMe_2	576	56,200	0.49 ± 0.02
11	Se	4-OMe	576	56,200	0.18 ± 0.09
12	Se	3-NH_2	582	56,300	−0.37 ± 0.092

[a] pH 6.0 phosphate buffer as the aqueous phase.

1 and **2**.[14,16] Dehydration with AcOH/HPF_6 gave the PF_6^- salts of dyes **3–12**. Ion exchange with Amberlite IRA-400 chloride exchange resin gave the chloride salts **3–12** in 40–98% isolated yields overall.

The chalcogenoxanthen-9-ones **1** and **2** have been prepared in low yield from benzamide derivatives **13** and **14**, respectively, and strong base.[14,16] We have found that the yields are greatly improved by cyclization with $POCl_3$ in refluxing acetonitrile as shown in Scheme 2. Chalcogenoxanthones **1** and **2** were isolated in 75% and 73% isolated yields, respectively, with this approach.

Values of absorption maxima, λ_{max}, and associated molar extinction coefficients, ε, as well as values of the *n*-octanol/water partition coefficient(log *P*) are compiled in Table 1. Values of λ_{max} are not very sensitive to the nature of the substituent attached to the 9-phenyl substituent on the chalcogenoxanthylium nucleus (Table 1). The range of values of λ_{max} for the thio **TMR** analogues is 565–573 nm, while the range of values for the seleno **TMR** analogues is 576–582 nm.

Values of the *n*-octanol/water partition coefficient, log *P*, are more sensitive to substituent effects with a range from −0.37 for 3-aminophenyl seleno derivative **12** to 1.13 for 3-*N*,*N*-dimethylaminophenyl thio derivative **6**. The *N*,*N*-dimethylamino-substituted derivatives **3**, **6**, and **10** and 4-methylphenyl derivative **5** were the most lipophilic dyes in the series. Methoxy-substituted derivatives **4**, **7**, and **11**, **TMR-S**, **TMR-Se**, and amino-substituted derivatives **8**, **9**, and **12** were far more hydrophilic with values of log *P* near 0.

Quantum yields for the generation of singlet oxygen [$\phi(^1O_2)$] by direct detection of singlet oxygen[17] and quantum yields for fluorescence (ϕ_F) were measured for **3–12** in $CHCl_3$ and MeOH and values are summarized in Table 2. Substituents on the 9-phenyl ring had a pronounced effect on values of $\phi(^1O_2)$ and ϕ_F. Values of both $\phi(^1O_2)$ and ϕ_F are low ($\leqslant$0.04) for derivatives containing the *N*,*N*-dimethylamino substituent, presumably due to increased rates of internal conversion in these systems. Even the seleno analogue **10** had a small value of $\phi(^1O_2)$, indicating the intersystem crossing was not competitive with internal conversion in these derivatives. Rates of internal conversion were not as dominant in the amino-substituted derivatives. Values of $\phi(^1O_2)$ were between 0.02 and 0.06 for thio derivatives **8** and **9**, while values of ϕ_F were between 0.01 and 0.09. The 3-aminophenyl seleno derivative **12** gave $\phi(^1O_2)$ of 0.31 in $CHCl_3$.

With **TMR-S**, **TMR-Se**, methoxyphenyl derivatives **4**, **7**, and **11**, and 4-methylphenyl derivative **5**, internal conversion was small relative to the combined yields of $\phi(^1O_2)$ and ϕ_F (Table 2). For the thio derivatives **TMR-S**, **4**, **5**, and **7**, $\phi(^1O_2)$ ranged between 0.09 and 0.30 and ϕ_F ranged between 0.23 and 0.44. For seleno derivatives **TMR-Se** and **11**, $\phi(^1O_2)$ ranged between 0.53 and 0.87, while ϕ_F was $\leqslant$0.01. In general, values of $\phi(^1O_2)$ were higher in $CHCl_3$ relative to MeOH, while values of ϕ_F were slightly higher in MeOH relative to $CHCl_3$.

2.2. Intracellular accumulation of heavy-chalcogen analogues of TMR

As shown in Table 2, thio analogues **TMR-S**, **4**, and **7** were sufficiently fluorescent to allow quantitative determination of cellular uptake into AUXB1 and CR1R12 cells. Drug-sensitive AUXB1 cells or multidrug-resistant CR1R12 cells were exposed to various concentrations of **TMR-S**, **4**, or **7** in the dark for 2 h in complete medium. The data displayed in Figure 1 demonstrate that nearly identical uptake is observed for **TMR-S**, **4**, and **7** in the drug-sensitive AUXB1 cells and that 5–8 femtomole per cell of these dyes is retained by the cells when exposed to 1×10^{-5} M dye for 2 h. Addition of 5×10^{-5} M verapamil prior to dye exposure did not affect the uptake of these dyes into the AUXB1 cells (data not shown).

In contrast, exposure of the multidrug-resistant CR1R12 cells to 1×10^{-5} M **TMR-S**, **4**, or **7** for 2 h gave only 3–4 femtomole per cell as shown in Figure 2. However, when the CR1R12 cells have a 15 min prior exposure to 5×10^{-5} M verapamil, a 2- to 4-fold increase in intracellular dye accumulation was obtained as shown in Figure 2 for dye concentrations of 1×10^{-6} to 1×10^{-5} M.

The data in Figure 2 demonstrate that uptake of **TMR-S** or dye **4** in CR1R12 cells is significantly greater for all the concentrations tested than the uptake of compound **7**. *P* values range from <0.05 to <0.001 for

Table 2. Quantum yields for the generation of singlet oxygen [$\phi(^1O_2)$] and for fluorescence (ϕ_F) in MeOH and $CHCl_3$ for **TMR-S**, **TMR-Se**, and **3–12**

Compound	9-Ph-X	$\phi(^1O_2)$		ϕ_F	
		MeOH[a]	$CHCl_3$[a]	MeOH[a]	$CHCl_3$[a]
TMR-S	H	0.21 ± 0.01[b]	—	0.44 ± 0.01[b]	—
TMR-Se	H	0.87 ± 0.02[b]	—	0.005 ± 0.01[b]	—
3	4-NMe_2	0.01 ± 0.01	0.01 ± 0.01	0.003 ± 0.001	0.003 ± 0.001
4	4-OMe	0.09 ± 0.01	0.22 ± 0.01	0.27 ± 0.01	0.23 ± 0.01
5	4-Me	0.11 ± 0.01	0.26 ± 0.01	0.29 ± 0.01	0.23 ± 0.01
6	3-NMe_2	0.02 ± 0.01	0.04 ± 0.01	0.001 ± 0.001	0.001 ± 0.001
7	3-OMe	0.16 ± 0.02	0.30 ± 0.02	0.35 ± 0.02	0.28 ± 0.02
8	4-NH_2	0.02 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.07 ± 0.01
9	3-NH_2	0.04 ± 0.01	0.06 ± 0.01	0.01 ± 0.01	0.09 ± 0.02
10	4-NMe_2	0.02 ± 0.01	0.02 ± 0.01	0.001 ± 0.001	0.001 ± 0.001
11	4-OMe	0.60 ± 0.02	0.53 ± 0.02	0.01 ± 0.01	0.01 ± 0.01
12	3-NH_2	0.05 ± 0.01	0.31 ± 0.01	0.004 ± 0.001	0.004 ± 0.001

[a] ±standard deviation.
[b] Ref. 14.

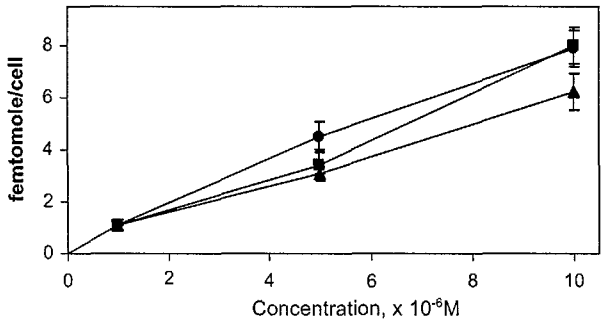


Figure 1. Intracellular accumulation of **TMR-S** (■) and thio derivatives **4** (●) or **7** (▲) with methoxy substituents into drug sensitive AUXB1 cells. Cell culture and dye exposure conditions and the method to determine the intracellular dye content are described in detail in Section 4. Each data point represents the mean obtained from at least three separate experiments performed in duplicate, error bars are the SEM.

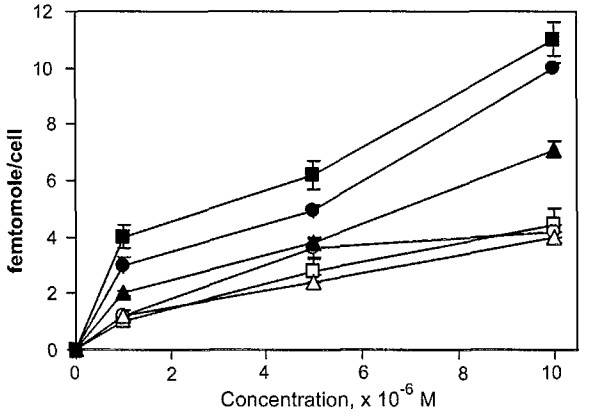


Figure 2. Intracellular accumulation of **TMRS** (□), **4** (○) or **7** (△) into cultured multidrug resistant CR1R12 cells and intracellular accumulation of **TMRS** (■), **4** (●) or **7** (▲) in cells exposed to 5×10^{-5} M verapamil prior to addition of dyes to cell monolayers. Cell culture and dye exposure conditions and the method to determine the intracellular dye content are described in detail in Section 4. Each data point represents the mean obtained from at least three separate experiments performed in duplicate, error bars are the SEM.

the comparisons. It is interesting to note that uptake of **TMR-S**, **4**, and **7** into CR1R12 cells after exposure to 5×10^{-5} M verapamil is equivalent to or marginally greater than the uptake of these dyes into the drug-sensitive AUXB1 cells (Fig. 1 vs. Fig. 2).

2.3. Phototoxicity of thio-analogues of TMR towards AUXB1 and CR1R12 cells

To compare the phototoxicity of the heavy-chalcogen analogues of **TMR**, cultured chemosensitive AUXB1 and multidrug-resistant CR1R12 cells were exposed to the compounds for 2 h in the dark and then irradiated with 5 J cm^{-2} broad band, 350–750 nm visible light. Dark controls were treated with dye for 2 h and not irradiated. The CR1R12 cells were treated with 5×10^{-5} M verapamil for 15 min prior to treatment with the thio analogues of **TMR**. The data shown in Figure 3 depict the results obtained from chemosensitive AUXB1 cells that were exposed to **TMR-S** and the thiotetramethylrosamine analogues **3–9**. In the absence of light, **TMR-S** and **3–9** gave >90% cell survival in AUXB1 cells at concentrations up to 1×10^{-5} M with or without added verapamil (data not shown). The data obtained demonstrate that **TMR-S** and 4-tolyl derivative **5** were the two most phototoxic compounds with nearly identical phototoxicity toward AUXB1 cells and were phototoxic at all the concentrations tested. 4-Methoxyphenyl derivative **4** was the next most effective photosensitizer and was significantly more phototoxic than 3-methoxyphenyl derivative **7** at lower dye concentrations. However, all four of these compounds have equivalent phototoxicity at or above 1×10^{-6} M. Derivative **3** is only phototoxic at concentrations of 1×10^{-6} M or higher. Analogues **6**, **8**, and **9** demonstrated little or no phototoxicity toward AUXB1 cells at any concentration. These data indicate that, at least at lower concentrations, substituent effects impact phototoxicity in the thio-tetramethylrosamine derivatives.

In the absence of prior exposure to verapamil, none of the thio analogues of **TMR** showed any significant dark or phototoxicity (>90% cell survival) toward CR1R12

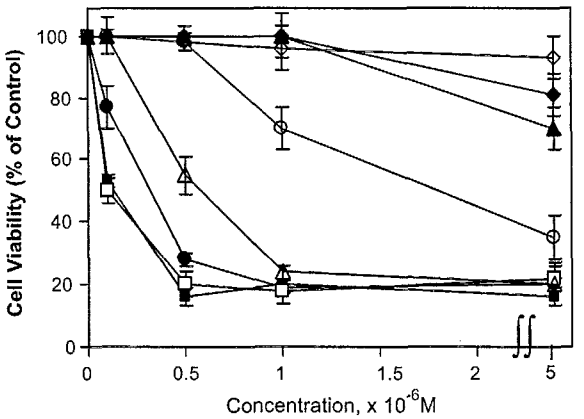


Figure 3. Phototoxicity of **TMR-S** and thio-tetramethylrosamine analogues **3–9** towards chemosensitive AUXB1 cells. The data depict the results obtained after chemosensitive AUXB1 cells were exposed to **TMR-S** (■), **3** (○), **4** (●), **5** (□), **6** (▲), **7** (△), **8** (◊), or **9** (♦). Cell culture and dye and light exposure conditions are described in detail in Section 4. Each data point represents the mean of at least three separate experiments performed in duplicate, error bars are the SEM. Data are expressed as percent cell viability compared to control cells, those exposed to neither dye nor light.

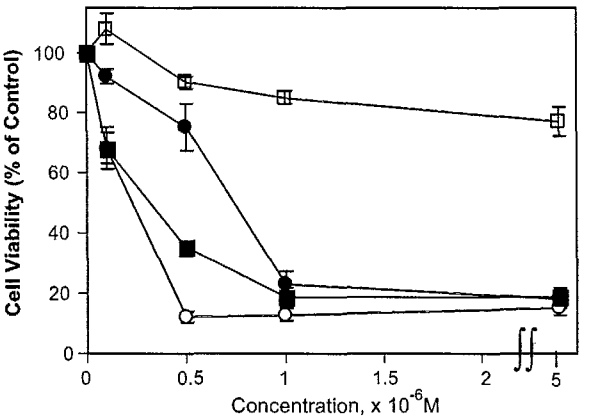


Figure 5. A comparison of the phototoxicity of **TMR-Se** (○) and seleno-tetramethylrosamine analogues **10** (●), **11** (■), and **12** (□). Cell culture and dye and light exposure conditions are described in detail in Section 4. Each data point represents the mean of at least three separate experiments performed in duplicate, error bars are the SEM. Data are expressed as percent cell viability compared to control cells, those exposed to neither dye nor light.

cells at concentrations $\leqslant 5 \times 10^{-6}$ M (data not shown). If the CR1R12 cells were treated with 5×10^{-5} M verapamil for 15 min prior to treatment with the photosensitizers, then substituent effects impacted photosensitizer potency. In the CR1R12 cells, none of the aminophenyl- or *N*,*N*-dimethylaminophenyl-substituted compounds—**3**, **6**, **8**, and **9**—showed any phototoxicity. However, the phototoxicity of **TMR-S** and the methoxy-substituted derivatives **4** and **7** toward these cells (Fig. 4) was consistent with their rank order of uptake: **TMR-S** > **4** > **7**, as shown in Figure 2. Derivative **5** was identical to **TMR-S** with respect to phototoxicity toward CR1R12 cells. In the presence of 5×10^{-5} M verapamil and in the absence of light, **TMR-S** and **3–9** gave >90% cell survival in CR1R12 cells at concentrations up to 1×10^{-5} M (data not shown). Comparison of cell viability following treatment of either AUXB1 or CR1R12 cells with **TMR-S**, **4**, **5**, or **7** and 5 J cm^{-2} of 350–750 nm light suggests that AUXB1 cells are more susceptible to photodamage than CR1R12 cells.

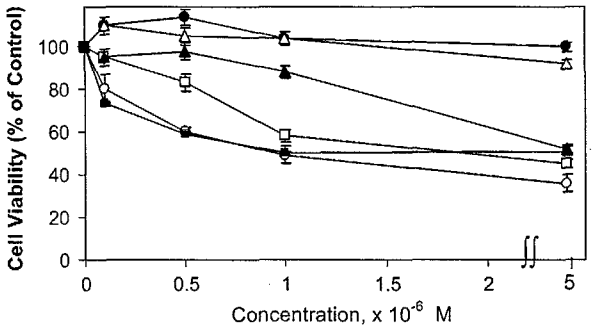


Figure 4. Phototoxicity of **TMR-S** (○), **3** (●), **4** (□), **5** (■), **6** (△), or **7** (▲) towards multidrug resistant CR1R12 cells following a 15-min incubation with 5×10^{-5} M verapamil. Cell culture and dye and light exposure conditions are described in detail in Section 4. Each data point represents the mean of at least three separate experiments performed in duplicate, error bars are the SEM. Data are expressed as percent cell viability compared to control cells, those exposed to neither dye nor light.

2.4. Phototoxicity of selenium-containing TMR analogues towards chemosensitive AUXB1 cells

The selenotetramethylrosamine analogues—**TMR-Se**, **10** with a 4-*N*,*N*-dimethylaminophenyl substituent, **11** with a 4-methoxyphenyl substituent, and **12** with a 3-aminophenyl substitutent—were tested for their phototoxicity toward chemosensitive AUXB1 cells with the data shown in Figure 5. **TMR-Se** and **TMR-S** (Fig. 3) displayed nearly identical phototoxicity as did thio analogue **4** (Fig. 3) and its seleno analogue **11** toward AUXB1 cells. The chalcogen atom had little impact on phototoxicity in spite of its impact on the values of ϕ ($^1O_2$) for **TMR-S**, **TMR-Se**, **4**, and **11** and related compounds. In the absence of light, none of these derivatives showed significant dark toxicity (>90% cell survival) at concentrations up to 1×10^{-5} M.

3. Discussion

A variety of substituents are readily introduced at the 9-position of the 2,7-bis-*N*,*N*-dimethylaminoxanthylium core via the addition of aryl Grignard reagents to the chalcogenoxanthone precursors **1** and **2**. The simplest analogues in the series are **TMR-S** and **TMR-Se**—the thio and seleno analogues of **TMR**. From the data in Table 1, the aryl substituents have little impact on wavelengths of absorption and the absorption maxima are dictated by the chalcogen atom. The thio-**TMR** analogues have values of λ_{max} near 570 nm, while the seleno-**TMR** analogues have values of λ_{max} near 580 nm. The 9-aryl substituents do impact values of log *P*, the *n*-octanol/water partition coefficient.

The 9-aryl substituents exert a strong influence on photophysical properties. The dominant features of the excited state photophysics are fluorescence, intersystem crossing to the triplet, which can then generate singlet oxygen, and internal conversion. The *N*,*N*-dimethylamino group markedly increases internal conversion at the expense of fluorescence and intersystem crossing to the triplet (Table 2). The 9-phenyl substituent, 9-4-methylphenyl substituent, 9-4-methoxyphenyl substituent, and 9-3-methoxyphenyl substituent appear to have much smaller contributions from internal conversion. The excited state photophysics for molecules containing these groups is dominated by fluorescence and intersystem crossing for the thio analogues as reflected in values of ϕ_F and $\phi(^1O_2)$ and by intersystem crossing for the seleno analogues as reflected in values of $\phi(^1O_2)$ (Table 2).

The data expressed in Figures 3 and 5 can be extrapolated to give the effective concentrations for 50% cell kill with 5 J cm^{-2} of 350–750 nm light (EC_{50}) for AUXB1 cells and, from Figure 4, the EC_{50} for CR1R12 cells. These values, while approximate, are compiled in Table 3. On the basis of the EC_{50} values, the rank order of photosensitizer efficiency toward AUXB1 cells is **TMR-S** ≈ **5** > **TMR-Se** > **4** ≈ **11** > **7** > **3** with **6**, **8**, **9**, and **12** showing essentially no phototoxicity at 5×10^{-6} M. The corresponding rank order for values of $\phi(^1O_2)$ is **TMR-Se** > **11** > **TMR-S** ≈ **7** > **5** > **4** > **3**, which suggests that photosensitizer potency is not a simple function of $\phi(^1O_2)$. Toward CR1R12 cells, the rank order of potency is **TMR-S** ≈ **5** > **4** > **7** with **3** and **6** showing essentially no phototoxicity at 5×10^{-6} M.

For AUXB1 cells, the uptake of **TMR-S**, **4**, and **7** is identical, suggesting that the 7-fold differences in values of EC_{50} are due to factors other than uptake and photophysical differences. In the CR1R12 cells, the phototoxicity and uptake appear to be directly correlated for **TMR-S**, **4**, and **7**, which may relate to the propensity of these materials to be transported by Pgp.

From values of $\phi(^1O_2)$ summarized in Table 2, **TMR-Se** is far more efficient than either **TMR-S** or **5** for the generation of singlet oxygen, yet all three of these molecules have similar potency as photosensitizers. Furthermore, values of log *P* are nearly identical for **TMR-S** and **TMR-Se** (0.07 and −0.09, respectively) and these two molecules are far more hydrophilic than thio derivative **5** (log *P* of 0.81, Table 1). Consequently, phototoxicity does not appear to be a function of lipophilicity. Another pair of interesting comparisons is found in the 4-*N*,*N*-dimethylaminophenyl-substituted derivatives **3** and **10**. As shown in Table 2, values of $\phi(^1O_2)$ are low and nearly identical for these two molecules in solution yet seleno analogue **10** has an EC_{50} of 0.7 times 10^{-6} M toward AUXB1 cells, while EC_{50} for **3** is 4×10^{-6} M. Both of these molecules are more lipophilic than **TMR-S** and **TMR-Se**. Clearly, the photosensitizer performance is not simply a function of either log *P* or $\phi(^1O_2)$.

These data, taken together, demonstrate that substituent effects among the various thio and selenotetramethylrosamine analogues impact phototoxicity toward either AUXB1 or CR1R12 cells. In the CR1R12 cells, the addition of verapamil enhances the uptake of the various photosensitizers and the relative phototoxicity of **TMR-S**, **4**, **5**, and **7** in both AUXB1 and CR1R12 cells follow the same trend **TMR-S** ≈ **5** > **4** > **7**.

In an earlier report, we demonstrated that photosensitization of Pgp-expressing CR1R12 cells by **TMR-S** and **TMR-Se** enhanced the uptake of the Pgp transport molecule Calcein AM, indicating that these analogues do impact the function of this drug efflux pump.[15] The data presented here demonstrate that substituent effects among the various thio and selenotetramethylrosamine analogues impact their phototoxicity toward either chemosensitive AUXB1 cells or multidrug-resistant CR1R12 cells. The substituent effects for the **TMR** analogues were realized in drug-resistant CR1R12 cells only when the Pgp modulator verapamil was present indicating that the substituent effects extend to the interaction between the chemical structure of the analogues and binding/transport function of Pgp.

Table 3. Concentrations of photosensitizers **TMR-S**, **TMR-Se**, and **3–12** to give 50% cell kill (EC_{50}) of AUXB1 or CR1R12 cells with 5 J cm^{-2} of 350–750 nm light

Compound	E	9-Ph-X	$EC_{50} \times 10^6$		$\phi(^1O_2)$, MeOH[a]
			M(AUXB1)	M(CR1R12)	
TMR-S	S	H	0.1	1.0	0.21[b]
TMR-Se	Se	H	0.2	—	0.87[b]
3	S	4-NMe_2	4.0	>5.0	0.01
4	S	4-OMe	0.3	2.0	0.09
5	S	4-Me	0.1	1.0	0.11
6	S	3-NMe_2	>5.0	>5.0	0.02
7	S	3-OMe	0.7	5.0	0.16
8	S	4-NH_2	>5.0	—	0.02
9	S	3-NH_2	>5.0	—	0.04
10	Se	4-NMe_2	0.7	—	0.02
11	Se	4-OMe	0.3	—	0.60
12	Se	3-NH_2	>5.0	—	0.05

[a] See Table 2 for standard deviation.

4. Experimental

4.1. General methods

All the chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise noted. Cell culture media and antibiotics were obtained from Grand Island Biological (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Atlanta, GA, USA).

4.1.1. General procedure for the preparation of chalcogenoxanthylium dyes: preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(4-*N*,*N*-dimethylaminophenyl)thioxanthylium chloride (3). A mixture of *N*,*N*-dimethyl-4-bromoaniline (0.50 g, 2.5 mmol) and ground magnesium turnings (0.060 g, 2.5 mmol) in 5 mL of anhydrous THF was heated at reflux for 2 h and then cooled to ambient temperature. The resulting solution was then added to a solution of thioxanthenone **1** (0.140 g, 0.47 mmol) in anhydrous THF (5 mL). The reaction mixture was heated at reflux for 1.5 h, cooled to ambient temperature and then to 0 °C. Following this, acetic acid (3.0 mL) was added. Hexafluorophosphoric acid (60% by-weight solution in water) was added dropwise until a color change was observed. Ice water (50 mL) was added and the resulting precipitate was filtered. The precipitate was dissolved in a 50/50 mixture of ethanol/acetonitrile and stirred with Amberlite IRA-400 chloride exchange resin (0.500 g) for 1 h. The resin was removed by filtration and the ion exchange was repeated with fresh resin (2x). The solvent was removed and the resulting solid was recrystallized from acetonitrile/ether to yield 0.164 mg (80%) of **3** as a dark purple solid, mp >300 °C. ^{1}H NMR (500 MHz, CD_3CN) δ 7.55 (d, 2H, J = 9.5 Hz), 7.19 (d, 2H, J = 8.5 Hz), 7.17 (2, 2H, J = 2.5 Hz), 7.00 (d × d, 2H, J = 2.5, 9.5 Hz), 6.93 (2, 2H, J = 9 Hz), 3.21 (s, 12H), 3.06 (s, 6H); ^{13}C NMR (75.5 MHz, CD_3CN) δ 160.6, 154.4, 152.3, 144.9, 137.5, 132.0, 123.4, 120.2, 116.0, 112.4, 106.4, 40.9, 40.5; HRMS (ES) *m/z* 402.2005 (calcd for $C_{25}H_{28}N_3S^+$: 402.1998); λ_{max} (CH_2Cl_2) 564 nm ($\varepsilon = 1.00 \times 10^5$ $M^{-1}cm^{-1}$), λ_{max} (CH_3OH) 565 nm ($\varepsilon = 1.05 \times 10^5$ $M^{-1}cm^{-1}$). Anal. Calcd for $C_{25}H_{28}N_3SCl$: C, 68.55; H, 6.44; N, 9.59. Found: C, 68.57; H, 6.31; N, 9.61.

4.1.2. Preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(4-methoxyphenyl)thioxanthylium chloride (4). 4-Bromoanisole (0.935 g, 5.0 mmol), ground magnesium turnings (0.12 g, 5.0 mmol), and thioxanthen-9-one **1** (0.10 g, 0.34 mmol) were treated as described. Following the final ion exchange, the filtrate was concentrated and the crude chloride salt was recrystallized from acetonitrile/ether to give 0.10 g (70%) of **4** as a dark purple solid, mp >300 °C. ^{1}H NMR (400 MHz, CD_3CN) δ 7.42 (d, 2H, J = 9.6 Hz), 7.27 (d, 2H, J = 8.8 Hz), 7.20 (d, 2H, J = 2.4 Hz), 7.17 (d, 2H, J = 8.8 Hz), 6.99 (d × d, 2H, J = 2.4, 9.6 Hz), 3.91 (s, 3H), 3.21 (s, 12H); ^{13}C NMR (75.5 MHz, CD_3CN) δ 161.6, 161.3, 154.6, 145.1, 137.3, 131.8, 128.7, 120.2, 116.3, 115.1, 106.6, 56.3, 41.0; HRMS (ES) *m/z* 389.1684 (calcd for $C_{24}H_{25}N_2OS^+$: 389.1682); λ_{max} (CH_2Cl_2) 569 nm ($\varepsilon = 1.10 \times 10^4$ $M^{-1}cm^{-1}$), λ_{max} (CH_3OH) 569 nm ($\varepsilon = 9.8 \times 10^4$ $M^{-1}cm^{-1}$). Anal. Calcd for $C_{24}H_{25}N_2OSCl$: C, 67.83; H, 5.93; N, 6.59. Found: C, 67.68; H, 6.04; N, 6.47.

4.1.3. Preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(4-methylphenyl)thioxanthylium chloride (5). *p*-Tolylmagnesiumbromide (1.34 mL, 1.3 mmol, 1 M solution in ether) and thioxanthen-9-one **1** (0.10 g, 0.34 mmol) were treated as described. Following the final ion exchange, the crude chloride salt was recrystallized from acetonitrile/ether to give 0.136 g (98%) of **5** as a dark purple solid, mp >300 °C. ^{1}H NMR (400 MHz, CD_3CN) δ 7.45 (d, 2H, J = 8.0 Hz), 7.36 (d, 2H, J = 10 Hz), 7.23 (d, 2H, J = 8.0 Hz), 7.20 (d, 2H, J = 2.8 Hz), 6.98 (d × d, 2H, J = 2.8, 10 Hz), 3.21 (s, 12H), 2.49 (s, 3H); ^{13}C NMR (125.5 MHz, CD_3CN) δ 161.3, 154.4, 145.0, 140.6, 137.1, 133.8, 130.2, 130.1, 119.9, 116.3, 106.5, 41.0, 21.4; HRMS (ES) *m/z* 373.1736 (calcd for $C_{24}H_{25}N_2S^+$: 373.1738); λ_{max} (CH_2Cl_2) 569 nm, ($\varepsilon = 13.1 \times 10^4$ $M^{-1}cm^{-1}$); λ_{max} (CH_3OH) 570 nm ($\varepsilon = 9.5 \times 10^4$ $M^{-1}cm^{-1}$). Anal. Calcd for $C_{24}H_{25}N_2SCl$: C, 67.83; H, 5.93; N, 6.59. Found: C, 67.84; H, 5.86; N, 6.62.

4.1.4. Preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(3-*N*,*N*-dimethylaminophenyl)thioxanthylium chloride (6). *N*,*N*-Dimethyl-3-bromoaniline (0.50 g, 2.5 mmol), ground magnesium turnings (0.060 g, 2.5 mmol), and thioxanthen-9-one **1** (0.14 g, 0.47 mmol) were treated as described. The crude chloride salt was recrystallized from acetonitrile and a small amount of ether to give 0.154 g (75%) of **10** as a dark green solid, mp >300 °C. ^{1}H NMR (500 MHz, CD_3OD) δ 7.62 (t, 1H, J = 8.0 Hz), 7.52 (d, 2H, J = 9.5 Hz), 7.38–7.32 (m, 3H), 7.14 (d × d, 2H, J = 2.5, 9.5 Hz), 7.08 (br s, 1H), 6.98 (br s, 1H), 4.91 (s, 12H), 3.15 (s, 6H); ^{13}C NMR (75.5 MHz, 50/50 CD_3OD/CD_2Cl_2) δ 160.8, 154.5, 148.7, 145.3, 137.8, 137.2, 130.7, 123.0, 119.8, 116.9 (2C), 116.1, 106.2, 42.9, 40.8; HRMS (ES) *m/z* 402.1996 (calcd for $C_{25}H_{28}N_3S^+$: 402.1998); λ_{max} (CH_2Cl_2) 573 nm ($\varepsilon = 1.09 \times 10^5$ $M^{-1}cm^{-1}$), λ_{max} (CH_3OH) 573 nm, ($\varepsilon = 7.3 \times 10^4$ $M^{-1}cm^{-1}$). Anal. Calcd for $C_{25}H_{28}N_3SCl$: C, 68.55; H, 6.44; N, 9.59. Found: C, 68.19; H, 6.51; N, 9.41.

4.1.5. Preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(3-methoxyphenyl)thioxanthylium chloride (7). 3-Bromoanisole (0.935 g, 5.0 mmol), ground magnesium turnings (0.12 g, 5.0 mmol), and thioxanthen-9-one **1** (0.10 g, 0.34 mmol) were treated as described. Following the final ion exchange, the crude chloride salt was recrystallized from acetonitrile/ether to give 0.070 g (40%) of **7** as a dark purple solid, mp >300 °C. ^{1}H NMR (400 MHz, CD_3CN) δ 7.54 (t, 1H, J = 8.2 Hz), 7.39 (d, 2H, J = 9.6 Hz), 7.22 (d, 2H, J = 2.4 Hz), 7.18 (d × d × d, 1H, J = 1.0, 2.6, 8.2 Hz), 6.99 (d × d, 2H, J = 9.6, 2.4 Hz), 6.93–6.91 (m, 2H), 3.82 (s, 3H), 3.22 (s, 12H); ^{13}C NMR (75.5 MHz, CD_3CN) δ 160.7, 160.5, 154.5, 145.1, 138.1, 137.1, 130.9, 122.4, 119.6, 116.4, 116.0, 115.6, 106.7, 56.2, 41.1; HRMS (ES) *m/z* 389.1684 (calcd for $C_{24}H_{25}N_2OS^+$: 389.1682); λ_{max} (CH_2Cl_2) 572 nm ($\varepsilon = 9.6 \times 10^4$ $M^{-1}cm^{-1}$), λ_{max}

(CH_3OH) 571 nm ($\varepsilon = 8.7 \times 10^4$ $M^{-1}cm^{-1}$). Anal. Calcd for $C_{24}H_{25}N_2OSCl$: C, 67.83; H, 5.93; N, 6.59. Found: C, 67.76; H, 5.91; N, 6.71.

4.1.6. Preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(4-aminophenyl)thioxanthylium chloride (8). 1-(4-Bromophenyl)-2,2,5,5-tetraethyl-1,2,5-azadisilolidine (0.50 g, 1.6 mmol), magnesium turnings (0.040 g, 1.7 mmol), and thioxanthen-9-one **1** (0.119 g, 0.40 mmol) were treated as described. Following the final ion exchange, the the crude chloride salt was recrystallized from CH_3CN/ether to give 0.095 g (58 %) of a dark green solid, mp > 300 °C. ^1H NMR (500 MHz, CD_3OD) δ 7.68 (d, 2H, $J = 10$ Hz), 7.30 (d, 2H, $J = 2.5$ Hz), 7.12 (d × d, 2H, $J = 2.5$, 10 Hz), 7.09 (d, 2H, $J = 8$ Hz), 6.91 (d, 2H, $J = 8$ Hz), 3.29 (s, 12H); ^{13}C NMR (75.5 MHz, CD_2Cl_2) δ 153.80, 144.74, 137.44, 131.46, 120.07, 115.29, 114.71, 105.66, 40.84; λ_{max} (CH_3OH) 565 nm ($\varepsilon = 7.63 \times 10^4$ M^{-1} cm^{-1}); HRMS (ESI) *m/z* 374.1697 (Calcd for $C_{23}H_{24}N_3S^+$: 374.1685). Anal. Calcd for $C_{23}H_{24}N_3SCl$: C, 67.38; H, 5.90; N, 10.25. Found: C, 67.22; H, 5.95; N, 10.26.

4.1.7. Preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(3-aminophenyl)thioxanthylium chloride (10). 1-(3-Bromophenyl)-2,2,5,5-tetraethyl-1,2,5-azadisilolidine (0.50 g, 1.6 mmol), magnesium turnings (0.040 g, 1.7 mmol), and thioxanthen-9-one **1** (0.119 g, 0.40 mmol) were treated as described. Following the final ion exchange, the crude chloride was recrystallized from acetonitrile and a small amount of diethyl ether to give 0.088 g (54%) of the product as a dark green solid, mp > 300 °C. ^1H NMR (500 MHz, CD_3OD) δ 7.64 (t, 1H, $J = 7.5$, 8.5 Hz), 7.45 (d, 2H, $J = 9.5$ Hz), 7.38 (m, 1H), 7.36 (d, 2H, $J = 2.5$ Hz), 7.15 (m, 2H), 7.12 (d × d, 2H, $J = 2.5$, 9.5 Hz), 3.31 (s, 12H); ^{13}C NMR (125 MHz, CD_3OD, 45 °C) δ 160.9, 155.3, 145.8, 138.5, 137.5, 137.4, 131.2, 131.1, 124.4, 120.3, 120.0, 116.7, 106.8, 40.7; λ_{max} (MeOH) 571 nm ($\varepsilon = 3.04 \times 10^4$ M^{-1} cm^{-1}); HRMS (ESI) *m/z* 374.1695 (Calcd for $C_{23}H_{24}N_3S^+$: 374.1685). Anal. Calcd for $C_{23}H_{24}N_3SCl$: C, 67.38; H, 5.90; N, 10.25. Found: C, 67.05; H, 6.01; N, 10.04.

4.1.8. Preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(4-*N*,*N*-dimethylaminophenyl)selenoxanthylium chloride (10). *N*,*N*-Dimethyl-4-bromoaniline (0.23 g, 1.2 mmol), ground magnesium turnings (0.030 g, 1.2 mmol), and selenoxanthen-9-one **2** (0.10 g, 0.29 mmol) were treated as described. Following the final ion exchange, the crude chloride salt was recrystallized from CH_3CN and a small amount of ether to give 0.089 g (69 %) of **10** as a dark green solid, mp 251–252 °C. ^1H NMR (500 MHz, CD_2Cl_2)δ 7.68 (d, 2H, $J = 9.8$ Hz), 7.24 (d, 2H, $J = 2.4$ Hz), 7.14 (d, 2H, $J = 8.5$ Hz), 6.87 (d, 2H, $J = 8.5$ Hz), 6.84 (d × d, 2H, $J = 2.4$, 9.8 Hz), 3.24 (s, 12H), 3.09 (s, 6H); ^{13}C NMR (125 MHz, CD_2Cl_2) δ 153.5, 144.4, 136.5, 135.4, 129.5, 129.2, 128.7, 119.2, 115.1, 114.2, 105.4, 40.5; λ_{max} (CH_2Cl_2) 576 nm ($\varepsilon = 5.6 \times 10^4$ M^{-1} cm^{-1},) 580 nm (sh, $\varepsilon = 5.7 \times 10^3$ M^{-1} cm^{-1}); HRMS (ESI) *m/z* 450.1440 (Calcd for $C_{25}H_{28}N_3{}^{80}Se$: 450.1443). Anal. Calcd for $C_{25}H_{28}N_3SeCl$: C, 61.92; H, 5.82; N, 8.67. Found: C, 61.83; H, 5.95; N, 8.47.

4.1.9. Preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(4-methoxyphenyl) selenoxanthylium chloride (11). 4-Bromoanisole (1.62 g, 17.0 mmol), ground magnesium turnings (0.22 g, 8.0 mmol), and selenoxanthen-9-one **2** (0.15 g, 0.43 mmol) were treated as described. Following the final ion exchange, the crude chloride salt was recrystallized from acetonitrile/ether to give 0.12 g (70%) of **10** as a black solid, mp 245–246 °C. ^1H NMR (500 MHz, CD_2Cl_2) δ 7.51 (d, 2H, $J = 9.8$ Hz), 7.45 (d, 2H, $J = 2.7$ Hz), 7.21 (d × d, 2H, $J = 1.8$, 6.7 Hz), 7.11 (d × d, 2H, $J = 1.8$, 6.7 Hz), 6.83 (d × d, 2H, $J = 2.7$, 9.8 Hz), 3.26 (s, 12H), 2.92 (s, 3H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 157.4, 148.9, 134.3, 130.9, 130.0, 128.6, 128.1, 126.5, 113.5, 112.9, 111.6, 54.8, 40.7; λ_{max} (CH_2Cl_2) 576 nm ($\varepsilon = 5.6 \times 10^4$ M^{-1} cm^{-1}), 578 nm, (sh, $\varepsilon = 1.7 \times 10^3$ M^{-1} cm^{-1}); HRMS (ESI) *m/z* 437.1133 (Calcd for $C_{24}H_{25}N_2$ $O^{80}Se^+$: 437.1127). Anal. Calcd for: C, 61.09; H, 5.34; N, 5.94. Found: C, 60.94; H, 5.48; N, 5.95.

4.1.10. Preparation of 2,7-bis-*N*,*N*-dimethylamino-9-(3-aminophenyl)selenoxanthylium chloride (12). 1-(3-Bromophenyl)-2,2,5,5-tetraethyl-1,2,5-azadisilolidine (0.50 g, 1.6 mmol), magnesium turnings (0.040 g, 1.7 mmol), and selenoxanthen-9-one **2** (0.14 g, 0.40 mmol) were treated as described. Following the final ion exchange, the crude chloride was recrystallized from CH_3CN and a small amount of diethyl ether to give 0.070 g (17 %) of the product as a dark green solid, mp 291–292 °C. ^1H NMR (500 MHz, CD_2Cl_2)δ 7.66 (d, 2H, $J = 9.8$ Hz), 7.48 (d, 2H, $J = 2.1$ Hz), 7.35 (t, 1H, $J = 7.5$ Hz), 6.92 (d × d, 1H, $J = 1.5$, 7.4 Hz), 6.87 (d × d, 2H, J = 2.1, 9.8 Hz), 6.74 (d × d, 1H, $J = 1.5$ Hz), 6.62 (d, 1H, $J = 7.6$ Hz) 4.50 (br s, 2H), 3.29 (s, 12H); ^{13}C NMR (300 MHz, CD_3OD) δ 154.7, 147.2, 139.7, 139.6, 130.7, 122.0, 120.7, 118.6, 118.4, 116.1, 110.0, 40.7; λ_{max} (CH_3OH) 582 nm ($\varepsilon = 5.6 \times 10^4$ M^{-1} cm^{-1}); λ_{max} (CH_2Cl_2) = 582 nm, ($\varepsilon = 3.7 \times 10^3$ M^{-1} cm^{-1}); HRMS (ESI) *m/z* 422.1142 (Calcd for $C_{23}H_{24}N_3{}^{80}Se^+$: 422.1130). Anal. Calc for $C_{23}H_{24}N_3SeCl$: C, 60.46; H, 5.29; N, 9.20. Found: C, 60.22; H, 5.51; N, 8.99.

4.1.11. Preparation of 1-(4-bromophenyl)-2,2,5,5-tetramethyl-1,2,5-azadisilolidine[18]. 4-Bromoanaline (1.00 g, 5.81 mmol), triethylamine (1.62 mL, 11.6 mmol) and 4-(dimethylamino)pyridine (0.070 g, 0.58 mmol) were dissolved in CH_2Cl_2 (20 mL) at ambient temperature. 1,2-bis(chlorodimethylsilyl)ethane (1.25 g, 5.81 mmol) in 5 mL of CH_2Cl_2 was added slowly and the resulting mixture was stirred for 3 h. Upon completion, hexanes (30 ml) were added to the reaction mixture precipitating triethylammonium chloride that was removed by filtration. The resulting pale yellow oil was purified by column chromatography on basic alumina, eluted with petroleum ether/ethyl acetate (4/1) to give the stabase-protected aniline in 1.66 g (91%) isolated yield: ^1H NMR ($CDCl_3$) δ 7.34 (AA′BB′, 2H, $J = 8.5$ Hz), 6.84 (AA′BB′, 2H, $J = 8.5$ Hz), 0.91 (s, 4H), 0.25 (s, 12H).

4.1.12. Preparation of 1-(3-bromophenyl)-2,2,5,5-tetramethyl-1,2,5-azadisilolidine[18]. 3-Bromoanaline (1.00 g, 5.81 mmol), triethylamine (1.62 mL, 11.6 mmol), 4-(dimethylamino)pyridine (0.070 g, 0.58 mmol), and 1,2-

bis(chlorodimethylsilyl)ethane (1.25 g, 5.81 mmol) were treated as described to give 1.66 g (93%) of a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.34 (AA′BB′, 2H, J = 8.5 Hz), 6.81 (AA′BB′, 2H, J = 8.5 Hz), 0.91 (s, 4H), 0.25 (s, 12H).

4.1.13. Preparation of bis-2,7-*N*,*N*-dimethylaminothioxanthen-9-one (1). Phosphorus oxychloride (2.7 mL, 29 mmol) and arylthiobenzamide **13** (1.08 g, 2.9 mmol) were heated at reflux in 50 mL of acetonitrile for 1.5 h and the reaction mixture was then poured into a mixture of 75 mL of 1 M NaOH and 75 g of ice. The resulting mixture was stirred for 1 h and the products were extracted with CH_2Cl_2 (3 × 50 mL). The combined organic extracts were washed with brine, dried over $MgSO_4$, and concentrated. The crude product was purified via recrystallization from CH_2Cl_2/hexanes to give 0.65 g (75%) of **1** as a yellow crystalline solid, mp 261–262 °C (lit.[14] mp: 260–261 °C): ^{1}H NMR (500 MHz, CD_2Cl_2) δ 8.42 (d, 2H, J = 9.2 Hz), 6.81 (d × d, 2H, J = 2.4, 9.2 Hz), 6.74 (d, 2H, J = 2.4 Hz), 3.11 (s, 12H), ^{13}C NMR (125 MHz, CD_2Cl_2) δ 177.2, 151.7, 138.6, 130.3, 118.5, 110.9, 104.8, 39.6; IR (KBr) 1589 cm^{-1}; λ_{max} (EtOH) 377 nm.

4.1.14. Preparation of bis-2,7-dimethylaminoselenoxanthen-9-one (2). Phosphorus oxychloride (2.4 mL, 26 mmol) and benzamide **14** (1.09 g, 2.6 mmol) were heated at reflux in 30 mL of acetonitrile for 1.5 h. Workup as described above gave 0.66 g (73%) of **2**, mp 224–225 °C (lit.[16] mp: 224–225 °C): ^{1}H NMR (500 MHz, CD_2Cl_2) δ 8.38 (d, 2H, J = 8.9 Hz), 6.80 (d × d, 2H, J = 8.9, 1.2 Hz), 6.75 (d, 2H, J = 1.2 Hz), 3.11 (s, 12H); ^{13}C NMR (CD_2Cl_2) δ 179.1, 151.6, 136.1, 131.6, 119.9, 111.0, 107.5, 39.5; IR (KBr) 1592 cm^{-1}; λ_{max} (EtOH) 388 nm.

4.2. Quantum yield determination for the generation of singlet oxygen

The quantum yields for the generation of singlet oxygen for **TMR-S**, **TMR-Se**, and **3–12** were measured by direct methods in methanol or chloroform.[19] A SPEX 270M spectrometer (Jobin Yvon) equipped with InGaAs photodetector (Electro-Optical Systems, Inc., USA) was used for recording singlet oxygen emission spectra. A diode-pumped solid-state laser (Millenia X, Spectra Physics) at 532 nm was the excitation source. The sample solution in a quartz cuvette was placed directly in front of the entrance slit of the spectrometer and the emission signal was collected at 90° relative to the exciting laser beam. An additional longpass filter (850LP) was used to attenuate the excitation laser and the fluorescence from the photosensitizer.

4.3. Fluorescence quantum yields

Fluorescence quantum yields (ϕ_F) were measured using techniques and equipment that we have previously described.[20]

4.4. Cells and culture conditions

Cultured cells used in this study were the Chinese hamster ovary parental cell line AUXB1,[21] a chemosensitive cell line that has a very low Pgp content and the multidrug-resistant subline, CR1R12, which highly constituitively expresses Pgp. Multidrug-resistant CR1R12 cells were established from the CH^{R}C5 cell line[22] by sequential culturing in increasing concentrations of colchicine with 5μg mL^{-1} being the final concentration used. Cell lines were maintained in passage culture on 60 mm diameter polystyrene dishes (Corning Costar, Corning, NY, USA) in 3.0 mL minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 50 units mL^{-1} penicillin G, 50 μg mL^{-1} streptomycin and 1.0μg mL^{-1} Fungizone® (complete medium). Only cells from passages 1–10 were used for experiments. A stock of cells, passages 1–4, were maintained at −86 °C to initiate experimental cultures. Cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere (Forma Scientific, Marietta, OH, USA). Passage was accomplished by removing the culture medium, then adding a 1.0 mL solution containing 0.25% trypsin, incubating at 37 °C for 2–5 min to remove cells from the surface, and seeding new culture dishes with an appropriate number of cells in 3.0 mL of complete medium. Cell counts were performed using either a hemocytometer or a particle counter (Model ZM, Coulter Electronics, Hialeah, FL, USA).

4.5. Determination of intracellular dye content

Cell lines, AUXB1 and CR1R12, were seeded on 96-well plates in 200 μL per well complete medium at 1–4 × 10^4 cells per well. Twenty-four hours after seeding, verapamil at 50 μM was added to selected wells in complete medium and cultures were incubated in the dark at 37 °C for 15 min. Dyes were then added to the cultures at various concentrations in complete medium. Cells were incubated in the dark at 37 °C for 2 h with or without verapamil in the presence of selected dyes. The medium was then removed and the monolayers washed once with 200 μL 0.9% NaCl and 200 μL 0.9% NaCl were added. The fluorescence of the intracellular dye was determined using a multiwell fluorescence plate reader (Gemini, Molecular Devices, Palo Alto, CA, USA). The excitation/emission wavelengths were set appropriately for each dye. Intracellular dye content was determined by comparing the magnitude of the fluorescence signal measured in each well to the fluorescence signal emitted from known concentrations of dye and calculating the femtomoles of intracellular dye per cell from those values.

4.6. Photoradiation of cell cultures

Cell lines, AUXB1 and CR1R12, were seeded on 96-well plates in 200 μL per well complete medium at 1–4 × 10^4 cells per well. Following treatment with or without 1 × 10^{-5} M verapamil, **TMR** analogues were then added directly to the cell culture medium at various concentrations and incubated for 2 h in the dark as above. The medium was then removed and 200 μL α-MEM minus FBS and phenol red (clear medium) were added to each well. One plate, with the lid removed, was then exposed to 350–750 nm light delivered at 1.4 mW cm^{-2} for 1 h (5.0 J cm^{-2}) from a filtered tungsten/halogen source, while a parallel plate was kept in the dark during the

irradiation period. Immediately following irradiation, the clear medium was replaced with complete medium and the monolayers were incubated for an additional 24 h period. Subsequently, cells were trypsinized and the MTT assay was used to determine cell viability. Data are expressed as percent cell viability compared to control cells which had been exposed to neither dye nor light.

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